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THE "BACTERIAL SYMBIOSIS" IN THE CONCRETION
DEPOSITS OF CERTAIN OPERCULATE LAND
MOLLUSKS OF THE FAMILIES CYCLO-
STOMATIDAE AND ANNULARIIDAE

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INTRODUCTION

Claparède described in 1858 a peculiar organ situated in the connective tissue of the dorsal region of the prosobranch mollusk, *Cyclostoma elegans*. This "glande" surrounding the intestines and boarding the nephridium consisted of snow-white, roundish granules. These had been noted as early as 1845 by Brard, who reported at that time that *Cyclostoma elegans* harbored a large quantity of small, "yellowish" calciferous granules, which were irregularly distributed throughout the connective tissue. Claparède made microchemical tests with the concretions, but failed to establish calcium as the main constituent. He was convinced that the organ had not been observed in other mollusks and designated it as "glande à concrétions." Garnault (1877) in the course of his careful studies on the anatomy and histology of *Cyclostoma elegans* devoted considerable attention to this "glande." According to this author the organ consists of a meshwork of closed tubules varying in length, but not exceeding 2 mm., the lumen of the tubules is filled with concretions, surrounded by clumps of rod-like bodies, which he recognized as bacilli. He attempted their cultivation by the methods, which had been introduced by Robert Koch about that time.

As recently as 1907, Barbieri discussing the concretions of *Cyclostoma* refused to accept Garnault's interpretation. Incidental to an histologic study of the embryo of this mollusk he made comments on the "glande à concrétions" and the rod-like elements, which it contained. He considered them to be small mineral concretions, probably phosphates. Unfortunately, no microchemical or bacterioscopic tests were reported to uphold his contention. The nature of these so-called bacteria was studied by Mercier (1911 and 1913) in connection with his work on the symbiotic micro-organisms of invertebrates. This investigator in a preliminary note, in 1911, accepted the view of Garnault and considered the rods of the "glande à concrétions" bacilli. He published, in 1913, his detailed studies and illustrated his observations by excellent plates. He showed in contrast to the findings of Garnault that the bacteria and concretions, which consisted of purine bases, namely uric acid, were not free in the tubules of a gland, but were enclosed in peculiar groups of cells located in the peri-intestinal connective tissue. These findings bring to mind numerous similar observations that have been made on bacterioids, mycorrhizas, mycetomas of plants and animals, and it is from this point of view that additional studies appear desirable.

M. Portier's treatise "Les symbiotes" (1918) and Buchner's (56) monograph "Tier und Pflanze in intracellulärer Symbiose" 1921, have recently aroused new

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interest in a relatively virgin field of investigation. Portier favors the conception that mitochondria are symbiotic micro-organisms. His contention is based on a very careful review of the literature, but his comparison between mitochondria and bacteria is lacking in directness. His interpretation has been upheld by his colleague Bierry and severely criticized on general grounds by Regaud, Guillermond and Laguesse. Auguste Lumière in his treatise "Le mythe des symbiotes" analyzes by means of bacteriological tests the claims of Portier. He concludes that the cells of normal animals do not contain micro-organisms, but that the tissues of vertebrates may enclose the spores of latent saprophytic bacteria. These will develop into active cultures when fragments of organs are transferred to nutritive mediums. The examples of symbiosis, which are observed in nature, are always evidence of a struggle between cell and parasite. There does not exist a true equilibrium between the two elements. Lumière concludes that mitochondria should not be confused either with bacteria or with the vitamins, which as chemical substances are poorly defined, and not identical with the symbiotes.

Meves (1918) has advanced the view independently of Portier that mitochondria are widely adapted symbiotic bacteria. He emphasizes the fact that one should always realize that the cell is the result of a progressive evolution.

Wallin (1922) has recently published observations on the staining of bacteria with mitochondrial methods and on the reactions of bacteria to chemical treatment. These have led him to advance an hypothesis similar to that of Portier, i. e., that all mitochondria are symbiotic bacteria. Whereas the evidence presented by Portier and Wallin is not convincing, it has a distinct bearing on the conception of the significance of the intracellular bacillary bodies in the concretion deposits of *Cyclostoma elegans*. If these views are correct, the identification of obscure intracellular elements cannot be established by accepted microscopical and bacteriological methods.

In this connection it appears appropriate to illustrate by a few examples selected from the literature the difficulties which have been encountered in deciding the exact nature and classification of the elements found in certain cells or groups of cells. A careful review of Buchner's book furnishes an endless list, but for the sake of brevity three interesting examples are given.

(1) The bacillary shaped organisms which are found in the root-nodules of leguminosae have received various interpretations. Beijerinck,¹³ Frank, Laurent and others were able to demonstrate that the rod-like elements are foreign to the plant tissue, may be cultivated in artificial mediums and may infect young plants grown on sterile soil. Until these facts were established the conception that these organisms were simple modifications of the cytoplasm had a number of adherents (Brunchorst, Vuillemin). (2) Blochmann reported in 1887 the presence of rod-like elements in the fat body cells of certain Blattidae (*Periplaneta orientalis*). He insisted on the great resemblance of these bodies to that of bacterial micro-organisms, and in 1892 declared them to be symbiotic bacteria, a view also shared by Forbes (1892). Subsequently, Cuénot (1892), Prenant (1904), Henneguy and C. K. Schneider concluded that the questionable inclusions were products of metabolism and not bacteria. These tiresome discussions were apparently put to end when Mercier (1906) announced the cultivation of the so-called "Blochmann bodies." He grew these organisms on nutrient agar, gelatine, potatoe, milk and bouillon and named them *Bacillus cuenoti*. Recent studies by Javelly, Hertig and Glaser support the original interpretation of Blochmann, but neither of the workers has thus far succeeded in propagating the true symbiote* on artificial mediums. The exact nature of the symbiotic

* Symbiont is a misnomer; the Greek word for "companion" or "partner" is symbiote.

organisms found in the fat body and the polar mass in the eggs of certain scale insects is equally uncertain. (3) The cellular elements, which have been observed by Buchner, Sulc and Pierantoni in the tissues of coccids and in other homoptera have usually been interpreted as yeast-like organisms, which are commonly referred to as the genus *Saccharomyces* or allied genera located in the same group of plants. Berlese (1905) has placed the organisms which he cultivated from Ceroplastes in the genus *Oöspora*, thus recognizing it as a true fungus (*Hyphomycetes*). Brues and Glaser have shown that the symbiote of *Pulvinaria innumerabilis*, which they succeeded in cultivating on artificial mediums cannot be regarded as a *saccharomyces*, but as a representative of the species "*Dematium*" or a related genus, although its morphology and method of multiplication in the insect are similar to those of a *saccharomycete*.

These examples will emphasize sufficiently the difficulties encountered in the identification of certain cellular elements. Furthermore, it is evident that morphological studies, staining reactions and the behavior toward chemicals are uncertain criteria in distinguishing intracellular metabolic products of animal and plant tissues from symbiotic bacteria, yeasts and fungi. Unstained specimens furnished the best information regarding the bacterial nature of intracellular bodies (Mercier, Buchner and others). This method of examination is unfortunately not always possible, and the investigator is dependent on fixed and stained preparations. Crystalloids, mitochondria, albuminous granulations, etc., present pictures, which are frequently mistaken for bacteria or yeasts. The following example cited by Mercier (1913, p. 7) needs no comment. Cuénot (85) observed certain connective tissue cells in the earthworm, filled with small colorless crystals. These were misinterpreted by Lortel and Despergins as "tubercle bacilli" ingested by the worm living in soil contaminated with sputum! Comparative morphologic study may, however, furnish valuable information. It is possible, in some cases, to follow the development of the crystalloids in the cells. Prenant, Limon and Legendre have pointed out that the crystals observed in the animal kingdom show inconstancy in form and arrangement, while the character of the intracellular bacteria and yeasts is remarkably constant. Opportunity will be afforded to discuss this statement in the course of this paper.

Microchemical tests furnish information of only relative value as Prenant pointed out in the sentence: "*la microchimie n'en est encorse qu' à la période d'essai.*" Crystals are frequently stained by aniline dyes; for example, those in the connective tissue of the earthworm take the Gram and the acid-fast stain as readily as tubercle bacilli (Cuénot, 1898). The crystalline formations in the liver of *Sphaeronia* and of *Gyge* observed by Bellonci and Emery are soluble in acid and alkalis. They are stained with iodine and therefore resemble the *Rhizobium* found in root-nodules of leguminous plants. Tests for solubility or the optical behavior of the bacillary intracellular elements in polarized or flourscopic light are rarely conclusive, as Mercier has pointed out in a number of excellent examples.

It must therefore be concluded that the propagation of intracellular bodies on artificial mediums constitutes the only decisive argument whereby they can be designated either as living micro-organisms, parasites or symbiotes. When yeasts or algae are present, the identification of the cultures is relatively easy. However, enormous difficulties may arise when the symbiotes belong to the polymorphic group of bacilli or bacteria. Under these circumstances it is important to test the physiological activities of the isolated organisms and to prove their parasitic or symbiotic properties by experimental inoculations. Such a procedure has been employed with great success by Noel Bernard, Magrou and others in the study of the fungi involved in the tuberization of various plants like orchids

and *Solanum tuberosum*. Such a mode of experimentation is, however, impossible when the infection and transmission of the symbiotes is maintained by an hereditary process. According to Blochmann, Henneguy, Mercier, Lindner, Sulc, Pierantoni, Buchner and others, most of the symbiotes infecting the insects are transmitted from generation to generation through the egg in a very definite manner. Even bacteria, which exist in the leave-nodules of *Ardisia crispa* are transmitted through the seed according to Miede. The hereditary transmission of the symbiotic micro-organisms explains their constant association with every individual of a particular species of host, but nullifies any attempt to test the cultivated micro-organisms by inoculating a host already contaminated through the egg.

The symbiotes from a small number of host species have been propagated on artificial mediums, but in most cases the cultivation experiments have met with unsurmountable difficulties. This may be due to the fact that the exact physiological conditions existing in the cells, the mycetomes or the bacteriocytes are still unknown. Analyzing the available literature, so ably summarized by Buchner, one realizes that the bacteriologist and the mycologist have found new fields for investigation. One gains the impression that a careful study of intracellular symbiosis in invertebrates may fertilize the field of pathology and immunology. The recent observations of Buchner on the constant occurrence of symbiotes in the mycetomes of blood-sucking insects like culex, anopheles, pediculi, etc., may have a distinct bearing on the conception of the transmission of pathogenic micro-organisms by insects. Equally novel and fascinating are the studies by Pierantoni, Harvey and others on the relation of bacteria to animal light. In the latter field, particularly, carefully controlled bacteriological tests may suggest numerous new problems.

The study of the concretion deposits of *Cyclostoma elegans* presents a number of intricate problems. Although the investigation is not complete, it appears advisable to report on the following phases thus far completed:

(1) A verification of the microscopic findings reported by Claparède, Garnault and Mercier on *Cyclostoma elegans*.

(2) An anatomical and histological study of the concretion deposits and nephridium of *Cyclostoma lutetianum*, *sulcatum* and *mauretanicum* * *Leonia mamillare*, *Tudora putre*, *Adamsiella variabilis* and *Chondropoma subreticulatum*, *majusculus* and *dentatum*.

(3) Bacteriological studies, an attempt to cultivate the intracellular bacteria of *Cyclostoma elegans*, *lutetianum*, *sulcatum* and *Leonia mamillare*.

(4) A biochemical and serological study of the predominant bacteria isolated from *Cyclostoma elegans*.

(5) Physiological studies on *Cyclostoma elegans* and the function of the concretion deposits.

* Since the foregoing has been written, a few specimens of *Cyclostoma Olivieri* Sowerby (Lebanon, Syria) and *Tudorella ferruginea* Lamarck (Balearic Isles) have been examined. They also possess concretion deposits and purinocytes infected with bacteria. The detailed studies will be reported in a separate paper.

ZOOLOGICAL STUDIES

ANATOMICAL OBSERVATIONS ON THE CONCRETION DEPOSITS
OF *CYCLOSTOMA ELEGANS* DRAP.

The specimens of *Cyclostoma elegans* employed in this study were collected near Liestal (Canton Baselland, Switzerland), where a small colony, located on a sunny, tertiary hill formation, had been known to Dr. F. Leuthardt for a number of years. Additional specimens have been received from Geneva through the courtesy of Dr. Jules Favre, and from Mendrisio (Canton Tessin), Grenoble, Marseilles, Nîmes and Nice (France), through Mr. Emilio Balli (Locarno), Dr. J. Perrin, Faculté des Sciences, Prof. A. Vayssière, Faculté des Sciences, Mr. E. Margier and Commandant Caziot, respectively.

A total of about 425 mollusks of this species have been examined. An idea of the sizes of the *Cyclostomas* studied may be gained from figure 1 on plate 1. In the majority of specimens, the concretion deposits are visible by transparency through the thin shell. As a rule, the position is on the first coil just opposite the operculum. After removal of the shell the whitish granular deposits located behind the nephridium in the dorsal region attract attention. The size of the "glande" varies considerably, and for convenience sake the concretion deposits have been divided into three groups, which are described separately.

Small or Type 1 Concretion Deposit (Fig. 2).—The intestinal loop, just above the nephridium is lined on each side by a whitish, feathery band of concretions. The whitish bodies are located in the oedematous-like connective tissue and not only surround the intestinal loop completely, but spread from the stomach between the folds of the digestive gland. This type has been noted quite frequently in the Spring.

Medium or Type 2 Concretion Deposit (Figs. 3 and 5).—The band of granular deposits which accompanies the intestinal loop is broad and densely packed with concretions. The "glande" consists of follicle-like aggregations of white-grayish spherical bodies. It infiltrates the connective tissue irregularly or surrounds mufflike the S-shaped intestinal loop, which is distinctly visible. The size and the location usually distinguish the "gland" from the calciferous cells, which are diffusely distributed in the upper coils of the body.

Large or Type 3 Concretion Deposits (Figs. 4, 6, 7 and 8).—The dorsal region presents a chalky-whitish, square shaped mass of concretions extending over an area of from 5 to 8 millimeters. Only fragments of the intestinal loop are recognizable on dissection. It is usually impossible to free the intestines or the stomach from the dry, cement-like concretion deposits. The concretions as a rule consist of small and large spherical granules. This type may give to the inexperienced an impression of a definite organ.

These three stages in the development of the concretion deposits have been noted by Garnault and Mercier. Garnault believes in a seasonal occurrence of the different sizes; he writes as follows: "d'une facon générale, bien qu'il y ait à ce fait de nombreuses exceptions la glande était beaucoup plus développée chez les animaux observés à la fin de l'automne, que chez ceux qui avaient passé l'hiver." Mercier, who collected several hundred specimens, observed *Cyclostomas* of a certain size and age with enormous concretion deposits, while others of the same size and age showed a fine band-like "glande." *Cyclostomas* collected in the early part of May presented the same large, medium and small concretion deposits as those examined in the last days of October. The same investigator marked a series of mollusks late in autumn and kept them in a terrarium during the winter months. He reexamined the animals in the following spring, but he found no changes either in the

TABLE 1
SEASONAL DISTRIBUTION OF TYPES 1, 2 AND 3 CONCRETION DEPOSITS IN *CYCLOSTOMA*

Season		Type I		Type II		Type III	
		♀	♂	♀	♂	♀	♂
Spring.....	55	14	16	7	6	8	4
Summer.....	26	3	4	5	6	4	4
Autumn.....	69	22	8	5	9	22	12
Total.....	150	39	28	17	12	34	20
	♀, 90; ♂, 60						

development or in the size of the concretion deposits. Variations in the size of the "glande" seemed to be equally independent of sex and Mercier attributed the differences mainly to individual factors. Table 1 records the size of the concretion deposits and the sex of the 150 *Cyclostomas* obtained from the Liestal Colony.

The 55 active spring specimens were collected on April 14; the summer forms on May 5 and subsequently kept in a terrarium under conditions similar to those found in the colony at Liestal. The autumn specimens were dug out from beneath stones and roots on October 19. It is evident that the statement of Garnault is substantiated by the data presented in table 1, although the number of individuals examined is not large enough for statistical analysis. Fine bandlike concretion deposits are frequently recorded in the *Cyclostomas* collected in the spring, while large "glandes" are more often encountered in the autumn specimens. Early casual observations that males examined in the spring have

usually type 1 concretion deposits have been confirmed. These observations, which indicate that the mollusks possess less extensive concretion deposits after hibernation than after a period of great physiologic activity, deserve further investigation, because they are contrary to those made on other gastropoda. Krahelska has, for example, shown that the kidney of *Helix pomatia* is transformed during hibernation to a storage organ for uric acid concretions. The nephridium of these snails collected in spring after hibernation resembles the concretion deposits of *Cyclostoma* collected late in the autumn. Until the physiological significance of the purine concretions in *Cyclostoma* has been established, it is impossible to give an explanation for the individual differences, which mask the seasonal variations as previously pointed out by Mercier. Anybody who has worked with operculate prosobranch mollusks is familiar with their irregular habits of feeding, locomotion, etc. In a series of observations on marked (artificially colored) *Cyclostomas* it was noted that the males and females which were moving around freely and fed quite regularly possessed late in summer large concretion deposits. This may have been a mere coincidence. One cannot escape the impression that the concretion deposits are, probably, not only an accumulation of waste material, but also represent stored substances which may be synthesized and used during periods of extreme dryness and lack of proper food. This aspect of the seasonal differences in the size of the concretion deposits will be discussed in a later chapter of this paper (p. 84).

MICROSCOPIC FINDINGS ON THE CONCRETION DEPOSITS OF
CYCLOSTOMA ELEGANS DRAP.

Technic.—Fragments of the concretion deposits have been teased in the lymph collected from the posterior vessels of *Cyclostoma* or in buffered salt solution. Sections have been prepared from dissected specimens of the mollusks fixed in mercury bichloride—absolute alcohol—(Schaudinn's), in saturated mercury bichloride-acetic acid—(5%) and Zenker's-solution. The mercury—acetic acid-fixation gave the best result. The serial sections were coated with a thin film of collodium in order to prevent the floating of the concretions. They were stained with haemalum prepared according to the method of P. Mayer and counterstained with orange G, or with iron-hematoxylin or by the Giemsa-Wolbach method (Mallory and Wright, p. 393). The latter procedure gave by far the most instructive pictures. The photomicrographic illustrations are enlargements of pictures obtained with the Zeiss "Phoku" photomicrographic ocular. A 100 watt "Osram" lamp with a Zettnow or "Tartrazin" filter (1:200) was employed as a light source. The exposures were made on "Perutz-Silbereosin," "Afga-chromo-isolar" or panchromatic plates and the enlargements were made by a fixed focus enlarging box on "contrasty" Gevaert's "Ortho-Brom" or "Novo-Brom" glossy bromide paper.

A concretion deposit dissected from the connective tissue surrounding the intestinal loop and carefully teased on a slide reveals under low power magnification three characteristic elements; loose connective tissue fibers, large cells carrying either brownish glycogen granules or opaque, spherical bodies of varying size. The latter bodies prepared from type 1 concretion deposits may show a compound tubular arrangement. When examined under high power magnification the opaque bodies or the concretions are situated in large cells which are studded with bacteria-like particles. The fluid surrounding the tissues is likewise filled with rods which have been set free by the crushing of the large cells. The findings are quite in accord with those already described by Garnault (p. 53). The drawings prepared from an unstained preparation and shown by this writer on plate 3, fig. 21, convey the impression that the concretions are located in the tubules of a gland. Stained sections, however, reveal a different picture. In preparations colored with Manson's blue, (fig. 11 and drawing in fig. 12) the concretions are found irregularly distributed in the connective tissue of the intestinal loops, the stomach and the pouches of the digestive gland. They are, as a rule, arranged in small nests or plaques located directly below the integumentum or close to the basal membrane of the intestines. The plaques of cells with their inclusions may give the impression of glandular tubules cut transversely or longitudinally. This arrangement is attributable to the grouping of the concretions and their cells around lymph vessels or lymph lacunae. The tissues in the region of the concretion deposits is well vascularized (fig. 16), a fact previously observed by Garnault and later confirmed by Mercier.

Serial sections of numerous specimens have failed to reveal any communication with the nephridium, the intestines or the integumentum. The "glande à concrétions" of Garnault is not an organ morphologically well separated from the surrounding tissues by a "membrane au histe," but a perivascular agglomeration of large cells which contain the concretions. Mercier reached a similar conclusion, but retained the term "glande à concrétions" for convenience sake. The large cells (1) for which Mercier proposed the name of "cellules uriques," are intermingled with amoebocytes (2) and with connective tissue elements, some carrying glycogen, (3) others harboring yellow brownish pigment, (4) ("néphrocytes," according to Cuénot) while a few contain calcareous granular material ("Calciferous cells") (5). These five groups of cells are found in every *Cyclostoma elegans*, from the smallest to the largest

forms. It should be stated in this connection that some of the mollusks may occasionally reveal a certain demarcation of the concretion layers from the peri-intestinal connective tissue. A true basal membrane has never been noted, but the loose, honey-combed connective tissue of the "gland" is distinct from the dense layers of similar tissue supporting the organs. The five types of cells, the concretions and the bacillary cell inclusions will be discussed separately.

THE DEVELOPMENT OF THE "CELLULES URIQUES" OR "PURINOCYTES"

The large cells, which carry the concretions were described and designated "les cellules uriques" by Mercier (1911). As recent chemical analyses to be reported in subsequent paragraphs have shown, however, that the concretions contain less than 50 per cent. of uric acid, the term "uric acid cells" is misleading. The word "purinocytes" is herewith proposed and used throughout this paper as a substitute for the name "cellules uriques."

The accuracy of the description of the purinocytes as given by Mercier in 1913 has been fully confirmed, although it has been impossible to study their gradual development on sections of the same mollusk. The evolution, which may occur in the purinocytes, has been followed on specimens of *Cyclostoma sulcatum* and will be described later.

The purinocytes, as previously stated, are found in nests or groups usually carrying several small or one large concretion surrounded by clusters of bacillary, thread-like elements (fig. 21, plate 2). In the vicinity of these aggregations one observes cells (fig. 21) which contain neither concretions nor rods nor vacuoles with small or large concretions. The cytoplasm of these cells presents an alveolar structure; the nucleus is an ellipsoidal body with fine nuclear fibers, numerous chromatin granules and a very definite nucleolus. The nuclei of the purinocytes are of the same size as those of the nephrocytes and of the glycogen-carrying reserve cells. Their large clumpy chromatin elements stain very deeply and are readily distinguished from nuclei of the adjacent connective tissue cells. This type of purinocyte may show in its cytoplasm a large vacuole containing a variable number of spherical bodies from 1 to 4μ in diameter (fig. 21 *A*, *cond.*) or a large stratified concretion. The nucleus (*N*) of these cells presents frequently an irregular shape and a central condensation of the chromatin into 2 or 3 clumpy masses. A comparative study of the small and large intracellular bodies indicates that the core of the large concretions is identical with the small globules situated in the vacuoles. These cores have been

designated by Garnault the "centre primaires"; they frequently retain the dyes in partially differentiated sections (fig. 15). The observation of Mercier, that they possess an acid reaction readily demonstrable by the purple tinge, when treated with neutral red, has been verified.

These "primary centers" may remain isolated or may fuse; in every case they grow by the gradual, onion-shell-like apposition of concentric lamellae (figs. 13 and 12). The latter remain colorless, when stained with neutral red and correspond to the "centres secondaires" described by Garnault. The secondary centers in the course of evolution are surrounded by numerous concentric thick or thin layers of concretion material until they reach dimensions varying from 0.1 to 0.3 mm. in diameter. The concentric lamellation in zones of different thickness seems to indicate that the development of the concretions does not take place continuously; periods of intensive apposition are followed by those of rest or diminished functional activity of the cell. This interpretation of the irregular lamellation has been advanced by Garnault and Mercier; it is supported by similar observations made by Harting on the evolution of the calcospherites. The growth of the concretions is preceded by an enlargement of the purinocytes (fig. 21).

The cytoplasm usually shows at this stage an invasion with bacillary bodies, although large purinocytes harboring no concretions but heavily infected with rods have been seen in every section examined. Mercier has described the initial stages of invasion, which are said to take place at the portion of the cell opposite to that, at which the concretions are forming. A large series of sections stained according to Giemsa's method or with iron hematoxylin have been searched for cells with early bacterial invasion, but without success. The purinocytes of 10 specimens of *Cyclostoma elegans* showed without exception a diffuse invasion of the cytoplasm by bacillary elements (fig. 21). The majority of these cells consisted of one large concretion surrounded on one side by a thin strip of cytoplasm packed with bacilli and an ellipsoidal deeply stained nucleus. The symbiotic complex between the bacilli on the one hand and the tissues of the mollusks on the other hand is even at this stage firmly established.

The findings here reported leave no doubt as to the excretory function of the purinocytes. Secretion of purines, growth of the concretions, multiplication of bacteria and definite enlargement of the entire cell proceed simultaneously in young as well as in old *Cyclostomas*. Although a careful search was made for stages showing the multiplication of

purinocytes, neither mitotic nor amitotic divisions were noted.* It is evident, as far as present findings permit conclusions that the giant growths of the purinocytes is not accompanied by any hindrance to the mitotic division of the cells. The histological picture fails to demonstrate any striking analogy between the mycetocytes of insects and the plaques of purinocytes, although in both species of invertebrates the cytoplasm of the cells is heavily burdened by foreign material. There are some indications of either a distinct excretory or a partially disturbed cellular activity in the purinocytes as evidenced by the irregular shape of the nuclei and the peculiar, clumpy appearance of the chromatic material. Buchner, while studying the mycetocytes of insects and Burgeff analyzing the rhizomas of orchides, have shown that intracellular symbiosis may provoke a variety of abnormal reactions on the nuclei. Definite degeneration observed in the cells that have been invaded by true parasites has never been recorded. These facts threw some light on the mutual relationship between the bacillary bodies and the purinocytes. The invaders are confined to the cytoplasm. The deleterious action is localized and reduced to a small area, and their adaptation seems to have attained a high degree of indifference. The foreign inclusions are without any visible effect on the functions of the cells.

The Bacillary Cell Inclusions.—The bacillary elements in the purinocytes deserve careful description, interpretation and discussion. Garnault (1887) reported for the first time the existence of rod-like elements in the concretion deposits of *Cyclostoma elegans*. This investigator did not hesitate to interpret these bodies as bacilli. Mercier (1911 and 1913) accepted the view of Garnault, while Barbieri, in 1907, considered the rod-like cell-inclusions phosphate crystals without furnishing any definite reasons for his view.

It is the purpose of this chapter to present additional evidence in support of the conceptions of Garnault and Mercier. The proofs, which can be used to identify the bacillary bodies as vegetable elements have been presented and analyzed in the introduction. There is no doubt that the bodies are intracellular. Extracellular forms are only found when the purinocytes are crushed. Unstained hanging drop prepara-

* In a recent paper Buchner reports his observation on the mycetocytes of *Acanthia lecularia*. The process of mitotic nuclear division of the mycetocytes may be suddenly inhibited; it is followed by giant cell formation and amitotic nuclear multiplication. Similar findings have been published by Heymons for *Periplaneta* and Schröder for *Trialeurodes*. Buchner (1923, p. 250) attributes these cellular disturbances to the influence exerted by the symbiotes resident in the cytoplasm of the mycetocytes. To illustrate this point, he cites a number of interesting examples on microsporidium infections in fishes, etc.

tions obtained by teasing a small fragment of the concretion deposits in salt solution or lymph reveal a number of important facts. The rods are immotile, but may show some Brownian movement. The majority measure between $2\text{-}5\mu$ in length and 0.4 to 0.5μ in thickness; filaments, which have a length of more than 20μ as described by Mercier are rare. In fact, excellent specimens demonstrating the arrangement and size of the rods, obtained in preparations stained with "Cyanochin" according to Eisenberg, have shown long thread-like elements in exceptional instances only. Clusters of rods in diphtheroid-like arrangement, some parallel, some curved and some club-shaped as illustrated by photomicrographs 22, 23, 24, 25 on plate 3 have been noted in numerous mollusks obtained from various sections of Europe. The size of the rods as observed in the "Cyanochin" smears varies according to the individual snails, but the length rarely exceeds 5μ . The formation of clusters similar to that observed in the purinocytes is regularly noted in carefully prepared smears.

The rods remain as a rule unstained, while a few may show a protoplasm which has a faint rose tinge. These findings are characteristic for all gram-negative rods. Vital dyes, like brilliant-cresyl-blue and neutral red, stain the rods faintly, but uniformly; on prolonged exposure some of the bacilli may show deeply stained granulations. The bodies are very resistant to treatment with strong acids (hydrochloric, acetic, nitric and sulfuric) alkalies (NaOH , KOH and Na_2CO_3) or ether, alcohol, etc., facts previously established by Garnault. The rods are readily stained by the ordinary basic aniline dyes. Thin smears fixed by heat, alcohol-ether, alcohol-acetone or bichloride-alcohol and stained with Manson's methylene blue- or Giemsa-solution give by far the best preparations. The variations in size already referred to are also noted in the stained smears (figs. 17, 18 and 19). Metachromatic granules and diphtheroid arrangement of the rods are seen in methylene blue preparations. The bacillary elements fail to retain the Gram stain, and they are not acid-fast. A large percentage of the rods stain very faintly with thionine or with dilute carbolfuchsin. Capsule and spore formation have not been noted. These observations on smears have been verified in studies made on sections stained with the identical dyes. The crowded intracellular location of the rods makes a cytological study very difficult, but the findings differ very little from those made on smears. The intracellular bacillary elements are very long and densely interwoven, forming a mesh work of actinomyces-like filaments. The bacterioscopic studies leave little doubt as to the bac-

terial nature of the rod-like, intracellular elements of *Cyclostoma elegans*. At the time these examinations were made, the publications of Wallin "On the Nature of Mitochondria" and of Cowdry and Olitsky on "Differences Between Mitochondria and Bacteria" attracted attention. The possible contention based on these studies that the bacillary, intracellular elements in the purinocytes are mitochondria must therefore be investigated and if possible refuted.

Cowdry and Olitsky showed that Janus green B was a specific reagent for mitochondria when employed in a dilution of at least 1:100,000. The mitochondria in living lymphocytes of the rabbit surpassed all other organisms in their affinity for this dye. On the other hand, no bacteria examined by them were colored on the addition of Janus green in this weak dilution of 1:100,000. Only two, *Streptococcus haemolyticus* and *Bacterium pneumosites*, were stained in a concentration of less than 1:10,000. The same investigators showed that the mitochondria were in general less resistant than bacteria. For example, they were invariably destroyed by treatment with 95 per cent. alcohol and with Bouin's fluid (picric acid-formalin-acetic acid); they were preserved by fixation in Zenker's fluid with and without acetic acid, in 10 per cent. formalin, in sublimate-acetic, and in Schaudinn mixtures. The distinction between the solubility of mitochondria and bacteria was often very sharp. There was also a pronounced difference in staining reaction after preservation. For example, Giemsa's stain, which is perhaps the best for the demonstration of bacteria, colored mitochondria very little, if at all. Noticeable differences in morphology can also be recorded between mitochondria and bacteria. The observations of M. R. Lewis and W. H. Lewis on mitochondria in tissue cultures, clearly indicate that the plasticity and modifiability of these elements is far greater than that possessed by any bacterial microorganisms.

The numerous cases in which symbiosis occurs in nature have naturally led some biologists to ask if symbiosis is not a phenomenon of general significance, and perhaps essential, in living organisms. In this connection reference is again made to the hypothesis advanced by Portier. The mitochondria that are present in all plant and animal cells, though not cultivatable, are, according to Portier, nothing but symbiotes, the importance of their function having recently been revealed by Guillermond, Dubreuil and others. For example, Guillermond has shown that the mitochondria of the epidermal cells in *Iris*

elaborates amyloplast and finally starch. Dubreuil found that mitochondria elaborate the fat in fat cells. Other cytologists have shown that glandular secretions are similarly referable to mitochondria. Portier applied his hypothesis to such varied problems as fecundation, parthenogenesis, tumor formation, variation, and origin of species, in all of which mitochondria, that is, his supposed symbiotes, play a part. His views aroused great controversy in France, so much so that it was thought necessary for the Société de Biologie de Paris to have a Committee examine the evidence (Compt. rend. Soc. de biol., 1920, 83, p. 654). The Committee, consisting on the one part of Portier and Bierry, and on the other of Martin and Marchoux, by its report indicates the pitfalls, well known to the bacteriologists, into which Bierry was led, and this disposes of the greater part of his far-reaching hypothesis. Quite recently Wallin (1923) has again approached the mitochondria problem experimentally. He claims to have "definitely produced independent growth of mitochondria in artificial culture media." Furthermore, he considered on theoretical grounds the evidence presented by Cowdry and Olitsky as inconclusive, and he maintains his conception of the bacterial nature of mitochondria. Keeping these facts in mind, a number of comparative tests on the bacillary elements of the purinocytes have been carried out.

Fragments of the concretion deposits were teased in a small amount of salt solution. A drop of this suspension was placed on a slide; to this was added a solution of Janus green B of known concentration in 0.85 per cent. saline solution. The pressure of the cover-glass brought about sufficient mixture of the fluids. In another set of experiments there was added a small amount of blood from the ear of a rabbit and the Janus green B solution of the same concentration, while in the third series, besides the rabbit blood, there was used a small drop of an emulsion of *B. coli* or *B. fluorescens* or tartar from the teeth with the same preparation of Janus green B. solution. By this method the reactions of the bacillary elements, of the mitochondria and of living bacteria within living cells were easily compared. The results are indicated in table 2.

TABLE 2
COMPARISON OF THE AVIDITY WITH WHICH MITOCHONDRIA IN LIVING CELLS, INTRACELLULAR BACILLARY ELEMENTS OF THE PURINOCYTES AND BACTERIA TAKE UP JANUS GREEN B

Concentration of Janus Green	Mitochondria		Bacillary Bodies of Purino- cytes	<i>B. coli</i>	<i>B. fluor- escens</i>	Mouth Bacteria
	Cyclos- toma	Rabbit				
1:1000.....	+	+	+	+	+	+
1:5000.....	+	+	+	+	+	+
1:10,000.....	+	+	±	+	+	+
1:20,000.....	+	+	—	(±)	(±)	+ streptococci
1:50,000.....	+	+	—	—	—	+ streptococci
1:100,000.....	+	+	—	—	—	± streptococci
1:200,000.....	—	+	—	—	—	— streptococci

The findings are striking and conclusive. The mitochondria in the living cells of *Cyclostoma* (phagocytes, digestive gland cells) and in the living lymphocytes of the rabbit stain intensely in dilutions of 1:100,000, while the bacillary bodies liberated or located inside the purinocytes color very faintly if at all in a dilution of 1:10,000. The bacteria, *B. coli* and *B. fluorescens* are faintly tinged in dilutions of 1:20,000; the streptococci stain in concentration of less than 1:50,000. These results definitely indicate that the intracellular bacillary bodies of the purinocytes behave in the presence of Janus green B as bacteria and not as mitochondria. The living bacteria, as a rule, obtained from cultures exhibited greater affinity for the dye than the bacteria of *Cyclostoma*; it may be that these bodies are either dead or chemically changed by the intracellular habitat. The mitochondria of *Cyclostoma* cells resemble spherical bodies or short rods of varying size; they are considerably smaller than the bacillary elements of the purinocytes. The findings presented in table 2 are supported by the evidence secured in studying sections fixed and stained by various methods. These observations are summarized in table 3.

TABLE 3
MITOCHONDRIA AND BACILLARY BODIES IN *CYCLOSTOMA ELEGANS*

Fixation	Stain	Mitochondria	Bacillary Bodies
Zenker's fluid with acetic acid...	Giemsa-Wolbach.....	—	+
	Iron hematoxylin.....	+	+
	Fuchsine and methyl green...	+	+
Sublimate-acetic.....	Giemsa-Wolbach.....	—	+
	Iron hematoxylin.....	—	+
	Fuchsine methyl green.....	—	+
Schaudinn's solution.....	Giemsa-Wolbach.....	—	+
	Iron hematoxylin.....	—	+
	Fuchsine methyl green.....	—	+

The results reported in table 3 correspond with the findings of Cowdry and Olitsky; they indicate that the chemical constitution of the mitochondria is fundamentally different from that of the bacillary bodies. A comparison both in the living state and in permanent preparations leaves little room for doubt that the intracellular bodies in the purinocytes of *Cyclostoma* are bacteria. The cultural results suggest the possibility that the major portion of the intracellular bacteria are no longer living. A number of tests were made to verify this conclusion by the three methods available to distinguish dead and living cells, namely, Nyfeldt's treatment of the specimens with silver nitrate, Kayser's staining with methylene blue and fuchsine and Henrici's procedure using a

Congo red solution.) The latter method, which makes use of the principle employed by Breed and Brew in counting bacteria and the negative staining method of Benians, was tried repeatedly, but inconclusive results were obtained. Even control specimens consisting of heat killed bacteria or stool specimens gave unsatisfactory smears. The bacteria of the purinocytes remained unstained and appeared as white spots on a blue ground. According to this unconvincing and unreliable test, the bacterial cells were alive at the time of staining. Contrary results were secured with Nyfeldt's method. A fragment of the concretion deposit was crushed on a slide suspended under a cover slip in a 5 per cent. argenti nitricum solution, and immediately examined in a strong light. Most of the bacteria were faintly brownish and within 5 minutes appeared deep black. Even the intracellular organisms were colored black after an exposure of 10 minutes to diffuse day or concentrated electric light. About 98 per cent. of the bacteria in hibernating mollusks were silver positive, while in summer specimens from about 80 to 90 per cent. of the rods were stained. Control preparations with old and young dead and living cultures of *B. coli* confirmed the value of Nyfeldt's method. Dead cells are deeply colored, whereas the living cells are not. The results secured by the Nyfeldt method suggest that most of the intracellular bacteria of the purinocytes are dead. At any rate, the permeability of the cell membrane or its electrical charge differs from that of living, extracellular bacteria obtained from cultures. It is not unlikely that the symbiosis has mummified the organisms or partially autolyzed or impregnated their membrane with uric acid, which in turn reduces the silver nitrate (Schiff's test). A final explanation for this interesting observation can only be given when additional experiments have been completed.

The Concretions.—Claparède, Garnault and Mercier have fully described the morphological and chemical structure of the concretions, but the present study has revealed a number of additional facts.

The concretions examined in salt solution appear as spherical or oval bodies; the majority of them are opaque and measure between 0.02 to 0.10 mm. The opaque ones are white in reflected light. The diameter of the smallest, transparent concretions is less than 0.005 mm. They dissolve slowly on the addition of acetic and hydrochloric acid. The action of sulphuric and nitric acid is similar, except that considerable gas is liberated in the course of dissolution. The inside structure of the concretion is best shown (figs. 9 and 10, plate 2) by treating them in

a weak solution of ammonia or sodium or potassium hydroxide. After an exposure of a few minutes, the crystalline material dissolves first at the margins leaving a fine transparent substratum. The latter is composed of one or several spherical, central granulations of slightly brownish color. These bodies sometimes single, sometimes in groups are surrounded by a system of concentric layers; these in turn are covered onion-shell-like, concentric lamellae of various thicknesses. Occasionally forms are seen which are not surrounded by an outer layer of lamellae (fig. 13, plate 2). It is evident that the substratum is the only remnant of the concretion.

Mercier has observed a diffuse clearing of the large, opaque concretions in polarized light, while the small bodies show a brilliant peripheral zone composed of numerous small crystals. This layer of very small crystals reflects the light so intensely in comparison to the center that the entire concretion seems to be lined by a luminous ring. The concretions exhibit the phenomenon of the black cross when treated with strong sodium hydroxide; not only the center of the concretions shows the black cross, but also the small spherical bodies which form in the hanging drop. These findings, which are identical with those of Schoppe on the nephridial concretions of *Helix pomatia*, have been made on numerous type 1 and 3 concretion deposits. The concretions are inactive in fluorescent light.

The chemical composition of the concretions has been studied by a number of workers. Barfurth (1884) reported a positive murexide reaction, while Garnault examined the solubility of the concretions in acids and alkalis. Aside from traces of CO_2 and calcium phosphate, he demonstrated primarily uric acid. For example, the treatment with HNO_3 resulted in the liberation of CO_2 and N in practically the same proportions as with pure uric acid. The murexide reaction appeared positive, although not clean cut. On the addition of ammonia not a purple red, but an orange color was noted; the latter in turn changed to a wine red instead of a violet tinge on the addition of sodium hydroxide. Mercier, who reviews the analysis of Garnault, remarks in this connection "Quand on fait une réaction de coloration, celle-ci ne doit pas être à peu près, elles est ou elle n'est pas." He therefore investigated by modern methods the chemical composition of the concretions and found an explanation for the doubtful murexide reaction secured by Garnault. The concretions are composed of uric acid, which is however, partially masked by xanthine and hypoxanthine. On separation of the purine bases, Mercier obtained the predominant substance, which not only gave a clear-cut murexide reaction, but fulfilled all other identity tests of uric acid. The methods used for the separation and identification of the purine bases are fully described in his publication of 1913, and the reader is referred to the original text. The description of the method is sometimes vague; for example the procedure as given on page 21: "Le précipité mis en suspension dans l'eau est ensuite décomposé par l'acide sulfhydrique, puis on porte à ébullition," etc., may frequently lead to a decom-

position of the purines. He concludes that the concretions consist predominantly of uric acid, which is associated with two xanthine bases. The amount of xanthine exceeds that of hypoxanthine.

The preliminary chemical tests gave at times conclusive murexide reactions, at other times doubtful ones, so that only a few analyses were therefore made. I acknowledge with appreciation the cooperation of Prof. K. Spiro and his assistant, Dr. St. Gadiant, at the Biochemical Laboratory of the University of Basel and the assistance of his associate, Miss E. Wagner, at the Hooper Foundation.

Unfortunately, the number of specimens of *Cyclostoma elegans* was limited and many of the tests or methods* which seemed desirable could not be carried out on account of lack of proper material. The experiments, which gave fairly conclusive results are herewith detailed:

Experiment 1.—The concretion deposits of 128 *Cyclostoma elegans* were carefully removed from the adjacent tissues ground and shaken in cold salt solution. The suspension was centrifugalized at low speed and the turbid supernatant fluid, which contained clumps of bacteria and destroyed purinocytes, was carefully removed from the grayish sediment. A portion of the latter was carefully dried. The 0.1496 gm. of substance were completely dissolved in 50 c.c. of *n*/17 NaOH. This solution contained a total of 0.0414 gm. N₂, as determined by the Folin-Farmer (1912, 11) micro-Kjeldahl method, and 0.063 gm. of uric acid as estimated by the colorimetric method of Folin and Wu (Jour. Biol. Chem., 1919, 38, p. 459). Assuming that the total nitrogen was present in form of uric acid, a total of 0.1242 gm. of uric acid should have been determined. However, only 50.7 per cent. of the nitrogen was identified as uric acid.

A second portion of the sediment was dissolved in boiling water and filtered. The filtrate of 40 c.c. was mixed with 2.4 gm. of sodium acetate, 4.0 gm. of a solution of sodium bisulphite and 5 c.c. of a 10 per cent. solution of copper sulphate and the mixture kept at boiling point for 5 minutes. The flocculent precipitate of copper purine bases was washed colorless, suspended in hot water and then decomposed by the careful addition of a 1 per cent. sodium sulphide solution. The mixture was acidified with hydrochloric acid, heated to boiling, filtered while hot and washed with hot water. The filtrate of 30 c.c. was evaporated to dryness dissolved in 10 c.c. of concentrated sulphuric acid and then diluted to 50 c.c. with distilled water. Crystals separated on standing and were filtered off. They were later identified as uric acid by the murexide, the Schiff test and with Folin's reagent. The filtrate was diluted to 100 c.c., neutralized with NaOH and treated again with copper sulphate and sodium bisulphide. This filtrate was strongly acidified with hydrochloric acid, a trace of potassium chlorate added and then evaporated to dryness. A thin pale canary-yellow residue formed, which gave when touched with ammonium hydroxide a rose-red color (Weidel's reactions) suggestive for xanthine.

Experiment 2.—The large concretion deposits of six *Cyclostoma* were extracted with successive portions of boiling distilled water, filtered and made up to 100 c.c. This solution had the following characteristics: it gave (a) a positive murexide test; (b) a strong positive color reaction with the phosphotungstic acid reagent of Folin (uric acid); (c) a positive test with Nessler's

* For a complete review of the subject the reader is referred to the monograph on Nucleic acids by Walter Jones (1914). The following references may also prove of interest: Davis, A. R.; Newton, E. B., and Benedict, S. R.: Combined Uric Acid in Beef Blood, Jour. Biol. Chem., 1922, 54, pp. 595-599. Rogers, H.: Exposure to Light as a Source of Error in Estimating Uric Acid by the Folin and Wu Method, *ibid.*, 1923, 53, pp. 325-331. Graves and Kober: The Nephelometric Estimation of Purine Bases, Including Uric Acid in Urine and Blood, Jour. Am. Chem. Soc., 1915, 37, p. 2430.

reagent indicative of some ammonium compound, and (d) negative protein tests. The solution contained 6.69 mg. N₂ per 100 c.c.; of the total nitrogen 2.42 mg. or 7.24 mg. Ur. was in form of uric acid as estimated by Folin's method (Jour. Biol. Chem., 1922, 54, p. 153).

Ten c.c. of the solution was oxidized with manganese dioxide in neutral solution and evaporated. A portion of the residue when evaporated with nitric acid on a porcelain surface gave a bright canary-yellow color indicating the presence of xanthine. Attempts to separate the purine bases failed, probably on account of the low concentration of the solution.

Experiment 3.—The concretions of 5 *Cyclostomas* were treated in a similar manner as described in Experiment 2. The same reactions were obtained. The total nitrogen was 9.25 mg. N₂ per 100 c.c., and the uric acid nitrogen was 3.34 mg. N₂ or 10.0 mg. Ur. An identification of the other purine bases with the aid of various methods was not successful.

The three experiments indicate that uric acid is one of the purine bases present in the concretions. Mercier (p. 24) concluded from his chemical tests with concretions collected from several hundred snails (p. 21) that "l'acide urique prédomine de beaucoup." The percentage figures for uric acid determined in 3 different series are shown in table 4.

TABLE 4

THE RELATION OF THE URIC ACID NITROGEN TO THE TOTAL NITROGEN IN THE CONCRETIONS OF *CYCLOSTOMA ELEGANS*

Total Nitrogen, Gm.	Uric Acid, Gm.	Percentage of N ₂ = Ur.
0.0414	0.0630	50.7
0.00669	0.00724	36.1
0.00925	0.0100	36.1

It is evident from the figures in table 4 that only 36.1 to 50.7 per cent. of the total nitrogen extracted from the concretions has been identified as uric acid. No attempts were made to wash out the soluble salts for making total nitrogen determinations, and the figures therefore apply to the total nitrogen of the concretions and not to the purine bases precipitated with ammoniacal silver solution as practiced by Mercier. In this connection, it should be stated that slightly higher percentages for uric acid might have been secured if a larger number of specimens had been used. In addition to uric acid, xanthine has been identified as one of the purine bases present in the concretion deposits. Quantitative estimations are not possible either for xanthine or for hypoxanthine, guanine or adenine, although one of these purine bases has been recognized qualitatively in the concretion deposits of *Leonia mamillare*. It is evident that aside from purine bases other,

probably soluble, nitrogen-compounds are present in the purinocytes and the concretions. Urea could not be demonstrated by the xanthydrol-acetic acid reagent of Fosse in two separate experiments using 9 concretion deposits. Histologic studies, however, leave no doubt but that this compound may occur in the purinocytes. The positive reaction with Nessler's reagent suggests the presence of ammonium salts; a quantitative estimation was not successful. The excretion of ammonium salts by mollusks is not a new observation. Meckel and Nalepa suspected ammonium urate in the excreta of gastropoda, without presenting however, satisfactory chemical evidence. Lindemann found ammonia in the nephridial fluid of Eledone, and Fuerth estimated that $\frac{1}{5}$ of the total nitrogen eliminated by the emunctoria of the Octopus leaves the body in the form of ammonia.

THE "NÉPHROCYTES DE CUÉNOT" AND THE CALCIFEROUS CELLS
IN THE CONCRETION DEPOSITS

Peculiar large cells with brownish-yellow pigment are present in both stained and unstained preparations of the concretion deposits. These cells have been called nephrocytes; they occasionally form broad strands between the islands of purinocytes. Their shape may be very irregular, usually elongated. They resemble young purinocytes although their cytoplasm is filled with pigment-granulations. It is difficult in unstained preparations to distinguish these cells from the glycogen carrying connective tissue elements. The large spherical nuclei with a fine network of nuclear fibrils carry a few clumpy chromatin particles (fig. 14, plate 2). When stained with Giemsa solution, the pigment granules acquire a green-bluish tinge. The function of the cells is best determined by injecting the mollusks with a suspension of carmine or India ink.* The phagocytic function of the cells is proved by the engorgement of the cytoplasm with carmine or India ink granules in animals sacrificed at the end of 3-5 days. It should be emphasized that the nephrocytes are the only cells in the concretion deposits aside from the amoebocytes, which fix artificially injected pigments. These observations, previously made by Mercier, definitely indicate that the concretion deposits contain two types of excretory cells, the nephro- and the purinocytes. The first absorbs artificially injected pigments, while the second lacks this property, but has the capacity to excrete and store

* An excellent summary on vital staining reaction will be found in the monographic presentation by W. von Möllendorff.

purine bases. These facts are quite in harmony with the statement of Cuénot (1900) that "dans le très grand nombre de reins à carminate connus jusqu'ici; il n'y a pas une seule fois fabrication d'acide urique ou d'urate."

Very little need to be said concerning the calciferous cells. No difficulties as a rule are encountered in distinguishing microscopically the few cells which occurs in the concretion deposits.

THE AMOEBOCYTES AND THEIR PHAGOCYTIC ACTIVITY IN THE CONCRETION DEPOSITS

Mercier has recognized and carefully described the phagocytic activity of the amoebocytes on the purinocytes, the bacteria and even the concretions. The specimens of *Cyclostoma* examined in this study have shown various stages of phagocytosis as described by Mercier. In some specimens, practically no signs were noted, while in others every field gave indication of phagocytic activity in the plaques of the purinocytes. It was impossible to determine definitely the factors which stimulate and support the process of phagocytosis. No attempt is therefore made to correlate the findings, but merely to record the various stages thus far observed.

Two stages of phagocytosis have been most frequently seen (plate 4). First, the formation of nests or islands of phagocytic cells, amoebocytes, carrying clusters and clumps of agglutinated, partially digested bacteria and actually replacing a portion of the concretion cells (figs. 27, 28 and 29) and second, an invasion and partial digestion of concretions by these same amoebocytes (fig. 26). The bacteria of the purinocytes are phagocytized first. The cells responsible for this are akin to resemble the lymph cells found in the sinuses and lymph vessels around the concretion deposits. They are relatively small, rarely exceeding 15 to 20 μ in diameter, with large regular or lobulated or fragmented nuclei rich in chromatin (figs. 28 and 29). The cytoplasm is always free from basophilic, acidophilic or neutrophilic granulations. They correspond in every respect with the lymphocytes, leukocytes, etc., observed in mollusks and described by Cuénot and Quagliariello. It is frequently difficult to outline the individual cells in the islands of from 30 to 50 amoebocytes. This fact was emphasized by Siedlecki in his studies on the amoebocytes in the coelom of annelides. The ingested and agglutinated bacteria are confined to a large vacuole. It is impossible to verify the conclusion of Mercier that they are gradually digested.

There is little room for doubt that the micro-organisms are changed in their behavior toward dyes. Most of them are granular and stain poorly. The question, "What happens to the amoebocytes, which have ingested bacteria?" remains thus far unsolved. The majority of the cells remain in situ, where they form bands or areas of scar tissue (fig. 29, *C T*), containing a few pigment carrying amoebocytes. A few phagocytic cells have, however, been observed in the lymph sinuses. Mercier, who studied the fate of these cells by injecting the snails with suspensions of carmine mentions as a possibility that the amoebocytes are carried by the general circulation until they are evacuated through the integumentum. This phenomenon of "direct excretion" has repeatedly been noted by Bruntz, de Bruyne, Cuénot and others in the order of the lamelli-branchiata, occasionally also in the prosobranch mollusks and the cephalopoda. Mercier, however, concludes that in his experiments pathological conditions were produced by the heavy injection of insoluble particulate matter, which made the fate of the amoebocytes loaded with ingested bacteria uncertain.

In the second type of phagocytosis frequently seen in sections, the cells participate in the dissolution of the concretions (figs. 26 and 29). The section material has been carefully searched for evidence, which would decide the problem, "What factors promote the phagocytosis of the purinocytes and concretions?" Neither morphological nor tincorial changes suggest a chemical process as the means of promoting the chemotaxis of the amoebocytes. The concretions as a rule are evenly surrounded by a single or multiple layer of epithelioid cells, which correspond to the amoebocytes already described. Some of the cells carry ingested bacteria, while others penetrate deeply into the outer layers of the concretion. Rindfleisch has seen similar conditions in the phagocytic destruction of gouty tophi in human joints. Advanced stages of phagocytosis tending to disrupt the large concretions, as illustrated by Mercier in his fig. 7 on Planche 2, and compared by him with the osteoclasts of bony tissues, have not been observed. However, a condition not recorded by Mercier deserves consideration. It is impossible for technical reasons to determine from a study of the sections whether the amoebocytes attack the intact, opaque concretions or primarily those which have previously lost their purine bodies, so that there remains only the well-known transparent, stratified organic substratum. I succeeded in answering this question by staining certain sections with dilute toluidine blue of a series, in which phagocytosis of the concretions had been previously noted. The photomicrograph in

fig. 26 prepared from a section rapidly stained by this method shows distinct phagocytosis of a transparent concretion, perfectly free from any crystalline material. The fuzzy, granular outline of several concretions not surrounded by leukocytes suggests a process of uricolysis, which is apparently independent of these cells. Furthermore, this observation supports strongly the contention that the amoebocytes remove only the organic structure of the concretions previously freed from its purine bases by agents as yet unknown. Evidence which will be presented in subsequent paragraphs suggests that the bacteria of the purinocytes are probably responsible for the solution of the uric acid crystals. A careful search for traces of purines in the amoebocytes surrounding the opaque concretions has been made by means of the silver-hydroquinone method according to Bauer. Although some black stained granules, which may or may not be uric acid, have been seen in the nephrocytes and the nephridial cells, no amoebocytes with purine bases in their cytoplasm have been observed. The resorption of the concretions, already admitted by Garnault and proved by Mercier, is a two phasic process. The dissolution of the purine bases attracts the amoebocytes, some of them ingest the bacteria, while others remove the organic structure of the concretions. A small band or island of cicatricial tissue indicates the area in which the purinocytes and their concretions have been absorbed through phagocytosis.

The description of the histologic findings on *Cyclostoma* cannot be closed without a brief consideration of the nephridium and the mechanism of excretion in this mollusk.

THE NEPHRIDIUM AND THE HISTOPHYSIOLOGY OF EXCRETION

The nephridium of *Cyclostoma elegans* has been described by Garnault and later by Perrier. Some notes concerning the functions of this organ have been made by Barfurth in connection with his study of the concretion deposits. This investigator failed to demonstrate uric acid in the emunctorium. He emphasized a physiological similarity between the nephridium of *Cyclostoma* and that of mussels. According to the numerous studies summarized by J. Strohl in his excellent monograph "On the excretion in mollusks," the nephridium of mussels is said to be free from uric acid, although it possesses definite excretory function. The macroscopic and microscopic examination of numerous specimens of *Cyclostoma elegans* serves as a foundation for a discussion of the anatomical studies made on other representatives of the family Cyclostomatidae (Annulariidae) reported for the first time in this paper. According to Erlanger, Drummond, Sachwatkin and others, it is now definitely established that the nephridium of the Prosobranchiata belonging to the suborder of the Monotocardia corresponds to the left kidney of the Diotocardia. The emunctorium is located behind the mantle cavity on the dorsal side, directly under the integumentum. After the removal of the shell, it is recognized as a gray-

brownish or olive green, triangular or round organ bordered by the mid and end-gut, the pericardium and the connective tissue carrying the concretion deposits. The histologic structure is briefly as follows:

Numerous lamaellae carrying the glandular epithelium project from a layer of connective tissue directly below the external lining into nephridial cavity. The lamellae anastomize; they form small cavities or tubes, which again communicate with the main cavity, but they never reach the opposite side of the organ. The central nephridial cavity, therefore, is quite spacious. The connective tissue enclosing the nephridium and in most of the lamellae, small and large lymph or blood carrying lacunae, or sinuses are noted. The renal epithelium consists of a single layer of vesicular or granular cells. The findings of Garnault, who reported several layers of excretory cells, have not been supported by Perrier (p. 206), who writes regarding this point as follows: "Le cyclostome serait le seul cas où les cellules rénales occuperaient plusieurs couches. Il est à priori permis de douter d'une pareille exception, et l'examen des coupes montre en effet que le cyclostome ne diffère pas dans ce rapport des autres prosobranches."

The nephridial lamellae carry vesicular cells, in fact this type of epithelium is the only one observed in about 150 nephridia examined by me. Granular cells, definitely ciliated, are present in the vicinity of the nephrostom and in the renopericardial duct; they play a very subordinate rôle as excretory cells; they are mainly destined to assist in the removal of the urine. The vesicular cells have a cubical or cylindrical shape; the portion situated on the lamellae is always narrower than that projecting into the nephridial cavity. The latter part contains a large vacuole, while the basal part encloses in a strip of cytoplasm a nucleus with a small nucleolus. The vacuole either remains clear and unstained, or it may show a large brownish-yellow or greenish, irregularly shaped concrement, or it is filled with numerous small yellowish granules. The examination of sections for urea-xanthidrol crystals in the nephridia of 110 Cyclostomas collected during the spring, summer and fall has furnished interesting data, which deserve some consideration.

The nephridia of mollusks with oedematous-like type 1 or 3 concretion deposits are usually voluminous. The vacuoles of the excretory epithelium are colorless and large; they may harbor a few small concretions. Little or no urinary debris is found in the central cavity. These findings were made on summer and fall specimens. Coarse, clumpy, brownish concretions have been observed in the renal epithelium and nephridial cavity of Cyclostomas with type 1 concretion deposits collected early in the spring. Unfortunately, no quantitative estimation of the concretions and the development of the concretion deposits has been made, but the available data convey the impression that the absorption of the purine bases during the winter months is followed or accompanied by an active elimination of insoluble waste material in the nephridium. In this connection, it must be emphasized that neither Cyclostomas with type 2 or type 3 concretion deposits showed this striking accumulation of waste material in the emunctorium. The process is, therefore, not merely the result of an interrupted elimination of the nephridial products during hibernation, but it is in some way connected with the activities in the concretion deposits. Furthermore, the conditions are by no means analogous to those regularly found and described by Krahelska in the emunctorium of *Helix pomatia* after hibernation or starvation.

The demonstration of large, unstained vacuoles, with small concretions or no contents at all in Cyclostomas with succulent, oedematous-like concretion deposits, indicate the existence of two functions. First, Cyclostoma kept in a

moist environment eliminate considerable amounts of water through the nephridium, and second, a portion of the excreta is removed in a soluble state. Urea has been identified with the xanthidrol reagent as one of the excretory products. This interpretation of the histologic findings is supported by the observation of Bial, who recorded the development of large vacuoles in the nephridial cells of Pulmonata transferred from a dry to a damp environment. On the other hand, the studies of Krahelska indicate that the formation of vacuoles in *Helix pomatia* may be independent of the moisture content of the medium in which the mollusks are kept. Thus far no comparative studies on the periodic variations of the functional activity have been made on the nephridium of prosobranch mollusks. The few facts here recorded have been accidentally collected. They may assist in an explanation of the factors responsible for the development and absorption of the purinocytes with their concretions.

The excreta which accumulate in the nephridial epithelium are discharged into the nephridial cavity and into the renal fluid as small vesicles with a thin membrane enclosing the brownish or greenish concrement. This mechanism of elimination is generally known as the "vesicular form" of the "merokrinous" mode of excretion. For *Cyclostoma*, Garnault described the so-called "holokrinous" secretion; the entire nephridial cell with its vacuole is torn from the lamella and drops into the cavity. Perrier who reinvestigated the nephridium of *Cyclostoma*, has shown that the vacuole and not the entire epithelial cells are discharged. The cytoplasm enclosing the nucleus remains intact and continues to function. In some of the spring specimens carrying large concretions in the nephridial cells, I have seen a type of "defecative" elimination of the contents. These findings support the views of Krahelska that the mode of discharge from the vacuole depends on the quantity and consistency of the excreta. According to Strohl, it is not unlikely that one and the same cell may successively show a "vesicular" or "defecative" type of elimination.

The form and the manner by which the waste products of metabolism reach the nephridial cells has been carefully discussed by J. Strohl. He suggests three possibilities: (1) The products enter the nephridial cells directly from the blood in a soluble state, (2) or in form of solid particles, or (3) the waste material is first taken up by the amoebocytes, which deliver it to the excretory cells. The latter function unquestionably occurs in the nephridium of *Cyclostoma* (as for example in fig. 46A). An examination of the emunctorium at low power reveals usually several intensively stained areas at the end of a lamella. Such a zone shows a lymphlacuna with numerous amoebocytes, which are surrounded by connective tissue bands and a structureless membrane carrying the nephridial epithelium. Typical amoebocytes are found in the lymph vessel, the connective tissue as well as the epithelium. In the latter tissue, they surround the single cells and apparently discharge waste material in soluble or granular form. This view is supported by the following observations: (a) The nephridial cells of the areas invaded by the amoebocytes are particularly rich in excreta, in comparison to other zones on the same lamella; (b) nephridial cells are seen which harbor in their cytoplasm deeply stained or brownish granules similar to those present in some of the amoebocytes, which are attached to them. According to J. Strohl, it is known that these granules undergo chemical changes and are finally transformed into the typical large hematophilic concretions of the vacuoles. Numerous sections have been studied without obtaining definite information concerning this transformation.

In connection with the study of phagocytosis in the concretion deposits, a series of *Cyclostomas* have been treated with dyes; 0.1 to 0.2 c.c. of an equal mixture of a 1 per cent. solution of indigo-carmin and of lithium-carmin were

injected into the foot of the mollusks. In a number of experiments, a 1 per cent. solution of trypan blue was used. The animals were sacrificed from 2 to 30 days after the last injection; the tissues were examined macroscopically and microscopically in unstained preparations or in sections. The findings are summarized in table 5.

TABLE 5
ATHROCYTOSIS IN *CYCLOSTOMA ELEGANS*

	Indigo-carmin	Lithium-carmin	Trypan Blue
Nephridium.....	Vacuoles of emunctorial epithelium in fine needles surrounding concretions	Amoebocytes in lacunae of the lamellae; occasionally few emunctorial cells of the renopericardial duct	Amoebocytes and epithelium of nephrostom
Concretion deposits and purinocytes	Leydig's cells or néphrocytes de Cuénot concentrated in vacuoles and young amoebocytes	Leydig cells and amoebocytes
Digestive gland.....	Several groups of cells.....	Occasionally a few cells
Intestines.....	Content of end-gut.....	Content of end-gut

It is noted that in accordance with the findings of Cuénot, the nephridial epithelium carrying large vacuoles and brown-greenish concretions collects and eliminates indigo-carmin. All the vesiculated, emunctorial cells on the lamellae are true "indigo-athrocytes" in the sense of Burian (p. 302). The elective localization of the dye in the nephridium may be seen by the deep blue color of the organ in mollusks injected with the violet mixture. The indigo-carmin is rapidly removed from the body and is retained by the nephridium, while the carmin remains in the connective tissue of the coils, which appear pink for several weeks. The cytoplasm and the nuclei of the indigo-athrocytic nephridial cells usually show a bluish tinge. In two mollusks injected repeatedly with the dye mixtures a few "carmin-athrocytes" located in the renopericardial duct and carrying the red dye in acid vacuoles have been observed. Similar findings have been reported by Cuénot. He believes that the dye experiments explain the dual function of the nephridium of the Monotocardia, which is still separated in the Diotocardia (indigo-athrocytes in the right and carmin-athrocytes in the left nephridium). It is rather doubtful whether this interpretation is absolutely correct. Carmin-athrocytes are exceptionally rare in or between the renal epithelium; as a rule, they are found in the lacunae of the lamellae and are either amoebocytes or Leydig's cells. In fact, it is quite likely that the carmin-cells, which are not ciliated as described by Cuénot, are emigrating amoebocytes passing through the renal epithelium. Mollusks injected with trypan blue lend considerable support to this interpretation.

The concretion deposits have been carefully scrutinized for selective deposition of dyes. Indigo-carmin cells are entirely absent, but the Leydig cells, or the "néphrocytes de Cuénot" and the amoebocytes are regularly filled with red vacuoles. Young and old, infected and uninfected purinocytes are invariably free from dyes. The occasional finding of a concretion surrounded by amoebocytes filled with carmin may be mistaken for athrocytosis by a purinocyte, an examination under oil immersion however reveals the actual condition. It is definitely established that the purinocytes are neither indigo nor carmin nor trypan blue athrocytes. This fact deserves brief consideration.

The histophysiological findings presented in the preceding pages leave no doubt that the purinocytes collect waste material in soluble form from the lymph and blood and transform it into concretions. Any cell which, according to Burian, collects and transforms soluble substances into vacuoles, granules or concretions, acts as an athrocyte and possesses marked affinities for certain dyes. The same point of view has been expressed by Strohl (p. 591). In fact, it is generally believed that the intra vitam dye experiments on mollusks furnish valuable information regarding the normal excretory function of certain cells. For example, it is definitely established that the emunctorial cells of the mollusks are indigo-athrocytes; although the affinity for this dye is not indicative of any particular waste product. The nephridial cells of the mussels eliminate in all probability urea, those of the gastropoda purine bases, particularly uric acid, and yet in both species the cells act as indigo-athrocytes. In *Cyclostoma* the dye experiments have apparently failed to serve as indicators for the normal excretory processes; a group of cells with definite concretions are neither indigo nor carmine-athrocytes. Whether this deficiency is due to the presence of bacteria in the cytoplasm of the cells or due to other functional properties deserve further investigation. Opportunity will be afforded to study the phenomenon of athrocytosis in some of the snails belonging to the genus *Chondropoma*.

The discussion of the nephridium of *Cyclostoma elegans* cannot be closed without a brief consideration of a few chemical tests which were undertaken to verify the statements of Barfurth. It is recalled that this worker failed to find uric acid in the emunctorium of this mollusk. While studying a series of sections stained by the silver-hydrochinone method of E. Bauer, I noted that the concretions in the vacuoles of the nephridial cells were distinctly visible by a black granular coating. The concretions simultaneously stained in the same section were a deep black. It suggested itself that the substances which reduced the silver were purine bases. Through the critical studies of Policard and Lacassagne, it is, however, known that every renal cell contains protein substances capable of precipitating the silver. The microtinctorial silver test for uric acid can obviously not be considered conclusive. The following tests have therefore been carried out.

A number of carefully dissected nephridiums were repeatedly extracted in boiling neutral, distilled water. The extracts were either concentrated to a small volume by evaporation in porcelain crucibles and then tested with Folin's uric acid reagent, or they were evaporated to dryness and subjected to a murexide test. Eight of 10 nephridiums treated in this manner gave definite reactions, which leave no doubt that uric acid is present in this organ. Judging by the intensity of the color reaction obtained with the phosphotungstic acid reagent, it is evident that the amount of this purine base is very small in comparison to that commonly found in the concretion deposits. These tests, moreover, do not establish the location of the base, whether it is in the cytoplasm or the excreta of the nephridial cells or the amoebocytes. The histologic findings on sections treated with silver suggest the concretions as the carriers of this waste product. The two nephridiums which according to the two tests contained no uric acid belonged to a group of *Cyclostomas* with very dry type 3 concretion deposits. No attempt will be made to correlate these findings. They confirm the early reports of Barfurth, and they furthermore suggest definite, individual variations in the excretion of uric acid by the nephridium. Observations to be detailed later have established the presence of urea in the nephridium of one *Cyclostoma elegans*.

MACRO- AND MICROSCOPICAL FINDINGS ON THE CONCRETION
DEPOSITS OF *CYCLOSTOMA LUTETIANUM* BOURG.

The four living specimens of *Cyclostoma lutetianum* Bourguignat used in this study were collected in February in the Forêt de la Sainte Baume (Var); they were received through the courtesy of Mr. E. Margier of Nîmes (France).

The mollusks deprived of their shell showed chalky whitish type 3 concretion deposits, which could not be distinguished from those of *Cyclostoma elegans*. However, sections and smears showed some differences in the size and the arrangement of the concretions. Even under low power the formation of foci or nests of large, somewhat oval-shaped concretions surrounded by clusters of small spherical concretions (fig. 33 plate 4) attracted attention. Purinocytes with fully developed lamellated bodies serve apparently as centers for the aggregation of young excretory cells showing various stages in the formation of concretions. In principal, the process does not differ from that fully described for *Cyclostoma elegans*. Furthermore, the finer structures of the purinocytes and concretions are identical as far as could be determined. The concretions consist of purine bases; they are soluble in boiling water and give a positive Folin's phosphotungstic-acid and murexide test. Resorption of the concretions through phagocytosis has been observed in the sections of two specimens. Amoebocytes invaded the purinocytes, ingested some of the bacteria and surrounded the concretions. In one specimen large layers of cicatrized tissue have been noted. The concretions are all opaque, they are definitely lamellated, the small forms may even show radial striation as seen in the nephridial concretions of *Helix pomatia*. Every purinocyte carrying concretions contains clusters of rod-shaped elements, which have been identified as bacteria. The intracellular symbiotes stain readily with the ordinary aniline dyes in smears and sections. They are nonmotile, gram-negative and not acid fast. In smears prepared from carefully teased concretion deposits, they appear as long threadlike, curved and bent threads (figs. 34 and 35). They are usually 8-10 μ long and less than 0.5 μ wide and resemble fragments of an actinomyces granule. Indications of true branching have been noted. Metachromatic granules but no spores, have been observed. As a rule, a few rods stain more deeply than the majority. The intracellular bacteria of *Cyclostoma lutetianum* differ from those of *Cyclostoma elegans* only by their width.

The nephridium shows the same structure, same type of nephridial cells and the same histophysiological mechanism as described for *Cyclostoma elegans*. The intestinal tract harbors spirochetes and the infusorium, *Trichodinopsis paradoxa*, which is regularly found in the intestines of *Cyclostoma elegans*.*

MACRO- AND MICROSCOPICAL FINDINGS ON THE CONCRETION
OF *CYCLOSTOMA SULCATUM* DRAP.

A collection of 80 specimens of *Cyclostoma sulcatum* Drap collected in March and April, 1923, were obtained from southern France through the courtesy of Mr. E. Margier, Nîmes, and Professor A. Vayssière, Faculté des Sciences, Marseilles. The individual snails with their reddish-yellow shell are generally slightly larger than the oldest specimens of *Cyclostoma elegans* described in this paper. Observed in a terrarium, they exhibited greater activity than the other specimens kept in the same environment.

The concretion deposits are located on the first coil; they can be seen through the thin shell. As a rule, they are smaller and confined to a shieldlike area just behind the nephridium. On dissection, two types of deposits have been observed: (a) the majority of the concretions are large and conspicuous by their whitish color; a few brownish granules are, however, scattered between them (figs. 38 and 39). (b) There is an equal number of white and brownish concretions or a bandlike infiltration in the connective tissue (fig. 37). The latter type resembles the concretion deposits characteristic for *Leonia mamillare*. The organ extends diffusely around the intestines, the digestive gland and the stomach. No sexual differences in the size of the deposits have been recognized; in fact, they correspond to the type 3 "glande" of *Cyclostoma elegans*. A predominance of brownish granules in the deposits of specimens of *Cyclostoma sulcatum* dissected in the fall after a sojourn of six months in a terrarium has been observed. It is believed that this type of concretion is formed during the summer months as a result of active metabolism, while their infiltration with crystalline purine bodies takes place during hibernation.

A concretion deposit carefully dissected from the connective tissue and teased in salt solution or lymph reveals two types of concretions (fig. 45)—(1) opaque roundish bodies, already described for *Cyclostoma elegans* or *Cyclostoma lutetianum* and (2) transparent, small, brownish, irregular granules. The latter type may show a clumpy dark center and a broad, nonstratified outer zone, or it may consist of a deep brownish, granular body resembling the excreta seen in the nephridial cells of *Helix pomatia* or other Gastropoda. These con-

* The parasite has been identified with the aid of the detailed descriptions given by R. Issel.

crements are resistant against alkalis and acids; they consist of amorphous material. They probably form the core for the transparent and opaque concretions. Claparède has reported the finding of brownish concretions in *Cyclostoma costulatum* obtained from the Banat, while Garnault (p. 52) claims their presence in the same mollusk secured from Algeria. The latter investigator undoubtedly studied another species of the family of the Cyclostomatidae, in all probability *Leonia mamillare*. *Cyclostoma costulatum* has not been found in Africa. All the specimens of this mollusk assembled in the extensive type collection of Mousson kept in the Zoological Institute at Zürich came from the Caucasian Banat or Armenia and not from Algeria. The size of the brownish concretions varies between 100 to 250 μ in diameter; they never attain the dimensions of the opaque concretions, which may measure between 0.05 to 0.25 mm. The fluid surrounding the suspended concretions contains small groups or numerous single, rod-shaped elements, which have been identified as bacteria. They are immotile, always curved, sometimes ring or spiral shaped (figs. 42 and 43). Dividing forms are common. The sizes vary between 2 to 10 μ in length and 0.5 to 1.0 μ in width; they resemble the symbiotes of *Cyclostoma lutetianum*, but are slightly larger than the bacteria found in the purinocytes of *Cyclostoma elegans*. Staining with various aniline dyes gave the following results: dilute fuchsin: pale, a few deeply stained forms; thionine: reddish, faintly colored; Manson's and alkaline methylene blue: best stain; no metachromatic granules; Giemsa-Wolbach stain: excellent preparations showing characteristic shape and arrangement (fig. 40); Gram stain: discolored, negative; acid-fast stain: fuchsin not retained; not acid-fast, "Cyanochin" preparations present on the homogeneous, blue background numerous curved rods with a light, pinkish, retracted cytoplasm indicative of the gram-negative character of the bacteria. The same staining reactions have been secured with the intracellular bacteria demonstrated in sections (fig. 48). The bacteria are very resistant to treatment with strong acids and alkalis. A comparative study between the mitochondria and the intracellular bodies by the methods described in a preceding chapter leave little room for doubt that the rodlike elements of *Cyclostoma sulcatum* are gram-negative bacteria. Until quite recently the intracellular organisms have been considered tissue symbiotes, but the following observation may force a reconsideration of this interpretation.

On July 16, 1923, a series of 8 *Cyclostoma sulcatum* were dissected and their concretions studied microscopically. *One snail failed to show bacteria*. Typical purinocytes with an alveolar cytoplasm and fully developed opaque or transparent concretions, entirely free from intracellular bacterial bodies, were found in numerous unstained hanging drop preparations or in stained smears or sections. Immediately 16 living specimens of *Cyclostoma sulcatum*, still available in the terrarium, were dissected. Every one of them was found to be infected. Furthermore, the records, smears and sections of every specimen studied, representing a total of 68 individual mollusks were again carefully scrutinized for bacteria and found to harbor typical intracellular microorganisms. The bacteria-free specimen which was received in a small box together with 37 others from the same colony in the vicinity of

Marseilles represents the only uninfected *Cyclostoma sulcatum* thus far observed. It is not the place to discuss the significance of this finding, but it clearly indicates the necessity to determine on a large series the actual percentage of noninfected mollusks in a colony. Whether one is justified to designate the intracellular bacteria as symbiotes, or as commensals, or as parasites, will depend entirely on the outcome of these investigations.

Sections through the concretion deposits of 10 specimens examined reveal a picture rarely encountered in *Cyclostoma elegans*. A number of purinocytes with large stratified concretions enclose an area mainly composed of cells carrying small brownish concretions. These nests of excretory cells are clearly shown in figures 44 and 50. In each compact island, purinocytes in various stages of development can be studied. Cells with a vacuole, a clumpy concrement and bacteria; some with or without either of these inclusions are found side by side. As far as the finer structure is concerned, no difference can be noted between the purinocytes of *Cyclostoma sulcatum* and those of *Cyclostoma elegans*. The cytoplasm of the irregularly shaped cells is alveolar; the nucleus is elliptical or irregular and contains a number of dense chromatin clumps. The descriptions given by Mercier, which I verified, for the development of purinocytes in *Cyclostoma elegans* fully apply to the conditions recorded in *Cyclostoma sulcatum*, although the vacuoles contain rarely more than one primary concretion. These inclusions may retain the hematoxylin; they are surrounded by bands of organic material, which, colored by the Giemsa method, either stain faintly greenish or remain brownish. The absence of onion-shell-like lamellations is typical for these small concretions, which are apparently free from crystalline material. The opaque concretions show primary and secondary centers and originate in the usual manner already described for *Cyclostoma elegans*.

Sections stained by the Giemsa-Wolbach method may attract attention on account of two types of cellular aggregations present in one and the same field:

(a) Large star shaped arrangements of purinocytes enclose broad greenish tinged spaces (dissolved concretions). Some of the cells with vacuoles and brownish concretions are heavily packed with clusters of bacteria (fig. 47).

(b) Islands of pale cells with dark nuclei separating round spaces occupied by large, stratified concretions consist of purinocytes, which have a narrow strip of cytoplasm, either free from bacteria or infected with a few poorly or deeply stained micro-organisms (fig. 50).

A purinocyte engorged with deeply stained bacteria is shown in fig. 48, while two cells similarly magnified presenting on the left hand numerous intracellular bodies and on the right side a granular, bacteria-free cytoplasm are pictured in fig. 49. No histophysiological reasons could be seen which would explain the presence or absence of bacteria in the cells. It is, however certain that the large purinocytes surrounding opaque concretions are invariably infected, although the demonstration of the micro-organism in these cells is sometimes difficult. The intracellular bacteria may retain the dyes slightly. Such cells stain deeply with acid dyes (eosinophilic); they may for example be red in Giemsa or hematoxylin-eosine preparations. The usual signs of intracellular bacterial autolysis are absent, and it cannot be stated with certainty whether the organisms are dead or not. It is quite likely that the physical chemical properties indicated by the striking eosinophilia are responsible for

the tinctorial behavior of the few intracellular bacteria. The changes in the nuclei of the purinocytes are slight; they consist of an irregular shape and of a condensation of the chromatin as observed in *Cyclostoma elegans*. The examinations of concretion deposits of *Cyclostoma sulcatum* definitely prove the excretory character of the purinocytes in the absence of bacteria. They also lend support to the conception of Mercier that the cells are infected by continuity from those already invaded by bacteria; certain chemotactic properties of the cell and not the size or condition of the concretions exert the stimulus necessary to attract the parasites.

Several hundred sections of 10 specimens have been searched for indications of phagocytic activity in the concretion deposits. Scattered amoebocytes attached to the purinocytes or concretions have been seen, but islands of phagocytic cells or invasion and partial digestion of the concretions as observed and described for *Cyclostoma elegans* have not been found. Even the places of predilection, namely, the connective tissue areas near the nephridium or the outer zones of the deposits, have been free from phagocytic activity. Mollusks preserved immediately on receipt and others kept in the terrarium for 8 months have presented equally negative findings. Nothing definite is, therefore, known concerning the disposal of the concretions, the bacteria and the old purinocytes.

An extract of the concretions in boiling water gave a definite reaction for uric acid with the phosphotungstic acid reagent. The murexide and the Weidel's reaction were conclusive. The yellow-reddish residue changed promptly to purple on addition of a drop of ammonia. A drop of caustic soda gave a blue-violet color. A large number of transparent concretions were selected with the aid of a fine pipet and a binocular microscope. They were dissolved and tested separately. A solution in hot distilled water gave a faintly bluish color, with the Folin reagent. When evaporated to dryness in the presence of HNO_3 , a brownish sediment was formed, which gave a negative murexide reaction. These tests seem to indicate an absence of uric acid in the transparent concretions. Unfortunately, sufficient mollusks were not available to repeat the chemical analyses reported for *Cyclostoma elegans*. From a physiological standpoint, it is important to know, whether this type of concretion consists of guanine or xanthine, as indicated by the tests conducted with material secured from *Leonia mamillare*.

The nephridia present histologically the same structure as described for those of *Cyclostoma elegans* (fig. 46). Functionally all the vesicular nephridial cells can act as indigo-athrocytes, but those covering the walls of the renal cavity retain the dye in form of large crystalline clusters. These cells are slightly smaller than those situated on the main lamellae. They are cylindrical; the cytoplasm is relatively small and carries a large vacuole, which is distinctly eosino or orangophilic. Each vacuole contains numerous spherical granules, which may stain deeply with acid dyes. The cells, which possess large vacuoles with colorless excreta, present a thin area on the portion projecting into the lumen. This condition indicates the place through which the content of the vacuole will be discharged. Cells with empty vacuoles and an opening at the free end must be considered the final stage of the "defecative" form of nephridial excretion as originally observed and described by Krahelska in her studies on the renal function of *Helix pomatia*. Amoebocytes, which emigrate from the blood lacunae convey and transfer the waste material from the blood and the tissues to the nephrocytes. Fig. 46 A shows these lamellae with definite amoebocytic activity. The two types of conveyance of granular excreta from the amoebocytes to the nephridial cells, as noted in *Cyclostoma elegans* and *lutetianum*, are also present in *Cyclostoma sulcatum*.

In conclusion it is recalled that *Cyclostoma sulcatum* possesses concretion deposits with two types of concretions: the opaque, white and the transparent, brownish type. The cycle of the purinocytes and their concretions is readily studied in sections. The intracellular bacteria are gram-negative rods. In a series of 68 specimens, one individual mollusk free from intracellular bacteria has been found.

MACRO- AND MICROSCOPICAL FINDINGS ON THE CONCRETION DEPOSITS
OF *CYCLOSTOMA MAURETANICUM* PLY

A small collection of 30 specimens of *Cyclostoma mauretanicum* Ply obtained from Rar-el-Maden, Beni-Saf, Algeria, through the courtesy of Mr. M. P. Pallary, Oran-Eckmühl, were available for study. The snails collected during the month of December resemble *Cyclostoma sulcatum* in size and shape, although the shell is more grayish in color and relatively thick. The individual mollusks when transferred to a terrarium with dead leaves, mosses and moist earth exhibited great activity in the first few days after arrival. They were seen in the act of feeding, copulation, etc., at a temperature of between 10-15 C. Later they were found hidden under the moss several centimeters deep in the damp soil.

The location of the concretions can only be determined after the removal of the thick shell. The dissected mollusks reveal large mottled white-brownish concretion deposits, which are closely confined to the connective tissue of the intestinal loops above the nephridium. The curved, finger-like arrangement of the organ surrounding the intestines is noted in every snail (plate 10, figs. 77 and 78). As a rule, the deposits protrude slightly above the adjacent tissues. The concretions are slightly brownish and the large spherical bodies are invariably accompanied by numerous black-brownish concretions. Occasionally the large concretions are relatively rare; consequently, the deposits are recognized with difficulty. No sexual differences in the size or coloring of the individual concretions or the deposits have been noted. As a whole, the deposits resemble those of *Cyclostoma sulcatum*, while the color is similar to that of *Leonia mamillare*.

Fragments of the deposits teased in lymph reveal two types of concretions (fig. 79): (1) large, spherical, white-brownish, but opaque elements, and (2) small, spherical, oval or irregular brownish and granular bodies which may be opaque or transparent. The opaque concretions are indistinguishable from those seen in *Cyclostoma elegans* or *sulcatum*; their diameter varies between 0.15 to 0.4 mm.; in fact, they are usually slightly larger than those observed

in the deposits of *Cyclostoma elegans*. The proportion of the small to the large granules may vary in the individual specimens, but the small always predominate. The diameter of the small concretions varies between 15 to 20 μ ; their mulberry-like, irregular center stains very deeply with dilute neutral red, they resemble the concretions seen in the nephridial cells of gastropod mollusks. Strong acids (HNO_3) dissolve these concretions without the evolution of gas, while the opaque types show effervescence when treated with the same chemicals. The latter reveal distinct lamellation and radial striation when suspended in a 5% KOH solution. The lamellae of the outer zone are frequently denser than those close to the "primary centers." The physical and chemical behavior of the two types of concretions is the same as described for *Cyclostoma sulcatum*. The small irregular, partially transparent concretions are the precursors of the opaque. An extract of the concretions in boiling water gave a strong reaction for uric acid with the phospho-tungstic acid reagent. The murexide and the Weidel's reaction were equally conclusive, although the tests indicated the predominance of xanthine bases. Quantitative tests have not been carried out.

The fluid surrounding the concretions in a hanging drop preparation secured by dissecting a deposit reveals clusters or numerous single, rather large, rod-shaped elements, which have been identified as bacteria (fig. 79). They are immotile, slightly bent or curved (figs. 80, 81 and 82). The sizes vary between 0.8 to 1.0 μ in width and 5 to 20 μ in length; as a whole, they resemble the "symbiotes" found in *Cyclostoma elegans*, but they are slightly larger than the organisms found in the purinocytes of the various *Cyclostomatidae* thus far examined. Moist fixation in bichloride solutions preserved admirably the size and the shape of the bacteria (fig. 82). Staining with various aniline dyes gave the following results: dilute fuchsin: deeply stained forms; thionine: reddish to deep blue; alkaline methylene blue: deep blue; no metachromatic granules; Giemsa-Wolbach stain: deep purple color; Gram stain: discolored; negative; acid-fast stain; fuchsin not retained, not acid-fast. The same staining reactions have been secured with the intracellular bacteria demonstrated in fixed tissues (fig. 84).

Sections through the concretion deposits of 5 specimens examined reveal a picture remarkably similar to that noted in *Cyclostoma sulcatum*. Group of purinocytes carrying small brownish, irregular bodies with or without bacteria, are surrounded by a ring of narrow cells harboring the large opaque or partially dissolved concretions. These islands distributed through the connective tissue present as a rule a complete cycle of the purinocytes, their products and their bacterial invaders. The same histological findings as illustrated by figure 50 for *Cyclostoma sulcatum* have invariably been noted in the sections prepared from specimens of *Cyclostoma mauretanicum*. Most of the purinocytes enclosing in a large vacuole and "primary centers" are free from bacteria (fig. 84, *F*). The cells, which harbor small opaque concretions are invaded by the large "symbiotes."

Considerable difficulty is encountered in finding the outline of the purinocytes, which are the carriers of the very large opaque concretions (fig. 83). Furthermore, the demonstration of micro-organisms in the compressed or completely obliterated cytoplasm is difficult. In fact, in a number of sections the concretions appear to rest loosely in the connective tissue spaces. Occasionally, a large concretion is surrounded by a thin membrane which encloses a compressed, elongated nucleus. Whether the cells remained uninfected or whether the intracellular bacteria have been removed by phagocytes, cannot be determined with certainty. The 5 mollusks studied in sections revealed a few scattered

plaques of phagocytes spreading between the clusters of purinocytes. The cytoplasm of the phagocytic cells may contain partially digested micro-organisms; it is reasonable to assume that the same mechanism as found in *Cyclostoma elegans* is responsible for the disposal of the old purinocytes and the bacteria.

The emunctoria present histologically a structure typical for prosobranch mollusks. Physiologically the nephridial cells can act as indigo-athrocytes. Every snail revealed a number of nephrocytes with large vacuoles, which enclose a brownish-yellow, granular concrement.

In conclusion, it is emphasized that *Cyclostoma mauretanicum* possesses concretion deposits with two types of concretions. The purinocytes are infected by large gram-negative rods. The development of the "cellules uriques," the structure and the histophysiological appearance of the nephridium are the same as observed in *Cyclostoma sulcatum*.

MACRO- AND MICROSCOPICAL FINDINGS ON THE CONCRETION DEPOSITS OF *LEONIA MAMILLARE* LINK

A collection of 49 specimens of *Leonia mamillare* secured in February from Oran, Algeria, through the courtesy of Mr. Paul Pallary (Oran-Eckmuhl) furnished the material for a comparative study of the concretion deposits. The mollusks moved around in the terrarium even during the winter months. They ingested water, but they were never observed in the act of feeding, although the intestinal tube contained fragments of plant material mixed with dirt.

It is impossible to determine the location of the concretion deposits on account of the thick, whitish shell. After its removal, the organ is found behind the nephridium as a small rectangular, brownish, shield-like area (fig. 53), which projects slightly above the adjacent tissues (figs. 54 and 52). The concretion deposits may vary somewhat in size; they are confined to the connective tissue between the intestine, the digestive gland and the nephridium. Neither seasonal nor sexual differences in the size of the deposits have been noted. The variations in size have been so slight, that a classification into types as employed in the description of the deposits of *Cyclostoma elegans* has been found impracticable.

The mottled appearance of the concretion deposits is due to two types of concretions (fig. 59); (a) small, spherical or oval bodies containing roundish or irregular, granular, blackish centers, which are surrounded by a light yellow-brownish, nonlamellated, transparent outer zone; (b) whitish, spherical bodies, opaque in transparent light and indistinguishable from those seen in *Cyclostoma elegans* and *sulcatum*, but never exceeding a diameter of 0.01 mm. The pro-

portion of the white to the brown granules may vary in the individual specimens, but the brownish color regularly predominates. It is noteworthy that in unstained preparations at least 12 to 15 purinocytes may form a cluster or group.

The transparent concretions are dissolved in strong H_2SO_4 and HNO_3 without the evolution of gas; while the opaque types show effervescence, when treated with the same chemicals. Weak solutions of HCl or NH_4OH dissolve the transparent outer zone of the brownish concretions, but they leave the core untouched. The opaque bodies gradually clear up by the same treatment and present insoluble centers indistinguishable from those present in the transparent, brownish concretions. Dense lamellation, which is typical for the opaque concretions of *Cyclostoma elegans* is rarely, if at all, observed. Furthermore, each concretion possesses only one, in exceptional instances two, "primary centers," which are either spherical or clumpy, mulberry-like and identical with the cores of the transparent bodies. The question naturally arose: "Is the transparent concretion the precursor of the opaque?" A number of tests have been made to solve, if possible, this problem.

Examined in polarized light, some of the brownish concretions show a peripheral zone consisting of radiating crystals; an opaque, central body is surrounded by a vividly luminating ring. Very small bodies of the same nature are perfectly clear and show crystalline radiations throughout. Others, again, remain dark and opaque. On the addition of 40% sodium carbonate the small and large transparent and opaque concretions present the phenomenon of the black cross. The same anisotropism may be seen in the minute granules, which develop in the solution. The opaque concretions behave somewhat differently in polarized light than those of *Cyclostoma elegans*. Instead of becoming brilliant, when turned under the microscope, they show around the edges two or more radiating patches, while the rest remains dark. These observations indicate that the transparent brown, as well as the opaque concretions contain crystals on an organic matrix. It was of importance to determine the chemical nature of this substance.

Unfortunately, only 3 mollusks could be sacrificed for this purpose. Several hundred brownish concretions were selected with the aid of a pipet under the microscope and dissolved in boiling water. A portion of this solution was tested with phosphotungstic acid reagent, no color reaction was obtained. The remainder was concentrated to a small volume. A portion of this concentrated solution, slowly evaporated with dilute HNO_3 , left a yellow-brownish sediment, which failed to give a definite murexide reaction, but changed to a violet color on heating and to a reddish color, when touched with KOH . The remainder of the solution, when alkalized with NaOH and mixed with diazobenzene sulphonic acid (Burian's test) gave a red color. A similar number of opaque concretions were dissolved in boiling water. The Folin and the murexide reaction were strongly positive. The concentration of the solution was insufficient to determine the presence of other purine bases. From these tests, one must conclude that the transparent concretions contain purine bases, probably guanine or xanthine, but not uric acid, while the opaque concretions are mainly composed of the latter purine. Furthermore, it is for the first time conclusively shown that morphologically different concretions possess different chemical compositions. The existence of such a condition was indicated in the chemical tests conducted with the concretions of *Cyclostoma sulcatum*, although conclusive reactions were not obtained with the small transparent excreta.

In this connection, it is advisable to record the chemical tests made with the extracts of 5 emunctoria secured in boiling, acidulated, distilled water. The filtrates of this extract gave a faint, but distinct color reaction with Folin's uric

acid reagent, and a few fine crystals of urea-xanthidrol formed on the addition of Fosse's xanthidrol acetic acid mixture. The murexide reaction was negative, while Burian's test for purine bases with diazobenzene-sulphonic acid gave a faintly reddish tinge. The nephridia of *Leonia mamillare* apparently contain traces of urea and purine bases, in form of uric acid, xanthine or guanine. The tests are suggestive, but they should be repeated with larger quantities of concretions and emunctoria.

Sections prepared through the deposits show the two types of the concretions clearly (figs. 60, 61, 62 and 63). The peri-intestinal connective tissue, which is well vascularized, is divided into numerous septums by dense fibroblastic strands (figs. 62 and 63). Groups of concretions are definitely separated by fibrillae, which color deeply red when treated with the van Gieson stain. A superficial inspection gives the impression of an alveolar gland without any definite excretory duct. The septums are well developed in the regions below the integumentum and near the nephridium; they are never found in the vicinity of the intestines or the digestive gland.

As every section of 11 specimens of *Leonia mamillare* examined has shown this alveolar structure of the connective tissue carrying the concretions, it has been impossible to determine the origin and purpose of this arrangement. It is not unlikely that the fibers are either a part of the cicatricial tissues which replaces absorbed concretions, or the localized development of the "glande à concrétions" necessitates a denser substratum to carry the excreta. An answer to these and similar questions may be expected from an embryologic study or an examination of very young specimens of *Leonia*.

The transparent concretions are regularly found in the vacuoles of large cells, which possess all the characteristics of purinocytes. As a rule, one cell has only one concrement. The cytoplasm of the irregular shaped purinocyte is filled with bacteria; the nuclei are oval and rich in chromatin. Developmental stages of the purinocytes and their concretions, as seen in *Cyclostoma elegans* and especially in *Cyclostoma sulcatum*, are rare. The impression is gained that the mollusks possess fully developed concretion deposits, which undergo little or no change during captivity. The structure of the young purinocytes with small vacuoles and clumpy granular concretions is practically identical to that described for *Cyclostoma elegans*. As a rule, the transparent outer zone of the excreta is either dissolved in sections stained by the Giemsa-Wolbach method, or it is visible as a faint greenish halo. The center core remains brownish and unstained. The opaque concretions remain intact or are partially transparent; sometimes they are eosinophilic in the outer zones (fig. 64). A fine strip of cytoplasm containing a few bacteria surrounds these bodies.

Chromocytes and athrocytic nephrocytes are found throughout the entire deposits. The septums are regularly invaded by amoebocytes and phagocytic activity around the purinocytes is quite common, although islands and foci of these cells as seen in *Cyclostoma elegans* are rare (fig. 64). A type of cicatricial tissue carrying fibroblasts and partially disintegrated amoebocytes is not infrequent in some sections (fig. 64). This picture suggests to the pathologist the repair of an injury and not merely the replacement of a few purinocytes. Whether the injury is caused by the concretions or by the toxin of the intracellular bacteria, cannot be determined from the sections and no attempt is made, to give a definite explanation, until additional specimens have been examined. It is, however, emphasized that these connective tissue reactions have not been observed in the concretion deposits of other *Cyclostomatidae* described in this paper.

The purinocytes of *Leonia mamillare* harbor in their cytoplasm groups of rodshaped bodies, which are unquestionably bacteria. The organisms are immotile in unstained preparations. They are usually single or in pairs, never in clusters; dividing and curved forms are rare. The size is fairly constant and rarely exceeds 4μ in length and 1 to 1.5μ in width (fig. 55). The rods are thicker than those of *Cyclostoma elegans*. They resist treatment with weak alkalis and acids; they stain readily with the ordinary aniline dyes. They are gram-negative and not acid fast. In preparations stained with Manson's blue, they show clear vacuoles resembling spores (fig. 56). The spore-staining methods of Möller and Burke gave negative results; "Cyanochin" preparations present on a homogeneous, blue background single, straight or slightly curved, bulbous, thick rods, which may occasionally show plasmolysis in form of a faintly, pinkish tinged protoplasm. This behavior in the dye solution supports the other reactions, which classified the rods with the gram-negative microorganisms. A comparative study of the rodlike elements with mitochondria in Janus green B solutions leaves no doubt that the intracellular bodies behave like true "bacteria." In sections stained with thionine or Giemsa solution, the intracellular location of the bacteria is definitely established. However, the cells are frequently so crowded that single rods may be difficult to recognize (fig. 58). Each cell may harbor several hundreds of bacteria, and every specimen of *Leonia* is found infected.

The nephridium of *Leonia* presents the same general structure and histophysiological activity as that of *Cyclostoma elegans*. The lamellae pierced by large lymph lacunae and projecting into a spacious nephridial cavity carry one layer of excretory cells (fig. 65). The nephridial cells show a vesicular or defecative form of discharging large clumpy, brownish or small granular excreta. Two types of cells, as seen in the nephridia of *Cyclostoma sulcatum*, are also found in *Leonia*. Amoebocytes transfer the waste material to the nephrocytes.

The nephridial cells lining the cavity act mainly as indigo-athrocytes. The dye is crystallized in the vacuoles in form of long needles or threadlike mycelial masses. The vesicular cells located on the lamellae and containing large concretions rarely eliminate indigo- or lithium-carmines. The concretion deposits of *Leonia* behave like those of *Cyclostoma elegans* and *sulcatum*; ammonium- and lithium-carmines granules may be found in the nephrocytes of the connective tissues, but never in the purinocytes.

In conclusion, it is recalled that *Leonia mamillare* presents small, confined, shieldlike concretion deposits, which are divided into septums by dense connective tissue fibrils. The purinocytes form two types of morphologically and chemically different concretions. The transparent, nonstratified concretions free from uric acid, contain probably xanthine or guanine, and represent about 60 per cent. of the excreta in the deposits. The other 40 per cent. of the bodies are opaque and consist mainly of uric acid. Large gram-negative, intracellular bacteria are present in the majority of the purinocytes. The formation of cicatricial tissue between the purinocytes deserves additional investigation, as the inciting stimulus for the reaction is not understood.

THE DEMONSTRATION OF CONCRETION DEPOSITS IN AMERICAN OPERCULATE LAND MOLLUSKS OF THE FAMILY ANNULARIIDAE

The demonstration of concretion deposits invariably associated with intracellular symbiotes in snails belonging to the genus *Cyclostoma* and the subgenus *Leonia* of the Old World operculate land mollusks of the family Cyclostomatidae, suggested an examination of related American forms. Nothing is known regarding the occurrence of concretion deposits or symbiotes in mollusks existing in tropical countries but related to *Cyclostoma elegans*. Prof. J. Strohl during a visit in Washington kindly consulted Dr. Paul Bartsch, Curator of the section of mollusks, United States National Museum and secured from him the following specimens preserved in alcohol: *Tudora putre*, *Adamsiella variabilis*, *Chondropoma subreticulatum* and *majusculus*, *Cyclophorus herklatsi* Marts (Fushima Japan); *Neocyclotus seminudus* C. B. Adams (Hollymount, Jamaica); *Coelopoma japonicum* A. Ad. (Fushima Japan); *Glossostyla appendiculatus necidious* Meldoff (Mt. Halson; Mindora Philippines) and *Glossostyla validus apoana* Barth (Mt. Apo, Mindanao, Philippines). It is a great pleasure to acknowledge again the courtesy of Dr. P. Bartsch in furnishing this interesting comparative material.

The specimens which were packed in alcohol were carefully removed from their shells, inspected and then imbedded in paraffine. The sections stained poorly with haemalum; they were mostly colored with dilute Manson's blue and differentiated in acidulated alcohol. Unfortunately, the nature of the fixatives used is unknown, and most of the dissected mollusks were unsuited for finer histologic studies, but definite concretions and even bacteria have been noted in the following species: *Tudora putre*, *Adamsiella variabilis*, *Chondropoma subreticulatum*, *majusculus* and *dentatum*. The details of the findings are briefly as follows:

(1). *Tudora (Tudorellata) putre* (G).—Pfeiffer from Guantanamo Bay, Eastern Cuba (Figs. 66, 67 and 68). An aggregation of large grayish bodies located in the connective tissue behind the nephridium in the dorsal region of the first coil resemble a typical "glande à concrétion." The concretions are large, mostly oval shaped and beautifully lamellated; they are situated below the integumentum and spread between the intestines, digestive gland, etc. (fig. 67). Finer structures, particularly purinocytes, could not be determined in the sections. In several areas closely connected with the concretions, small clusters of large stumpy, partially disintegrated bacteria are seen. Single rods are frequent throughout the deposits, but not in the intestines or other organs. This location suggests a connection between bacteria and concretions.

(2). *Adamsiella variabilis*.—C. B. Adams from Hollymount Jamaica (figs. 69 and 70). The two specimens show large, greenish granules in the dorsal portion of the first coil and in the vicinity of the nephridium. Large oval shaped,

lamellated elements arranged in groups and surrounded by granular débris are seen in sections (fig. 70). Elements which resemble bacteria have not been demonstrated in properly stained preparations.

(3). *Chondropoma subreticulatum*.—Maltz, from Haiti (Figs. 71, 72 and 73). Three different snails present large and definite concretion deposits behind the nephridium spreading between the digestive gland and the intestines. Mostly lamellated, oval-shaped bodies were seen in sections. Between these concretions clumps of granular bodies, which stained deeply with dilute fuchsine or methylene-blue, attract attention. Properly magnified, these granules appear as clusters of spherical bodies resembling torula-like cells or very large cocci. They are only found in the concretion deposits. It is naturally impossible to express an opinion concerning the nature of these elements, without a study of living or properly preserved specimens

(4). *Chondropoma majusculus* Pfeiffer.—From Cuba (figs. 74 and 75). Whitish granules, which could not be distinguished from the deposits of the calciferous tissues are seen around and between the intestines and glands. Microscopically oval or spherical, lamellated bodies of varying sizes and indistinguishable from typical concretions are found in the connective tissue filling the peri-intestinal spaces. Neither bacteria nor yeasts are observed in properly stained sections. The nephridium presents a structure typical for the representatives of the family Cyclostomatidae.

As the fixed specimens of Annulariidae were unsatisfactory for finer cytological studies, an attempt has been made to obtain living mollusks. Through the courtesy of Mr. C. T. Simpson, Little River, Florida, four living specimens of *Chondropoma* (*Chondropomorus*) *dentatum* Say, and from Dr. O. Schöbel, Bureau of Science, Manila, two specimens of *Glossostyla appendiculatus* Meldoff have been received. An anatomical study has confirmed the findings made on preserved specimens, namely, *Glossostyla* has no concretions in the peri-intestinal connective tissue. The excretion of purines takes place through the nephridium, which possesses nephrocytes with striated concretions similar to those commonly found in the emunctoria of the Helicidae. However, the structure of the *Glossostyla* nephridium presents a number of interesting conditions, which will be presented and discussed in a separate paper. The findings on *Chondropoma dentatum* supplement the observations made on *Chondropoma subreticulatum* and *Chondropoma majusculus*. They are considered of considerable importance in connection with the study of concretion deposits in mollusks. A detailed account is therefore given.

(5). *Chondropoma* (*Chondropomorus*) *dentatum* Say.—From Florida, U. S. A. The mollusks are in size and arrangement of the coils similar to *Chondropoma subreticulatum* (plate 14, fig. 71). Deprived of their shell, they show bands and aggregations of whitish concretions extending from the nephridium on the first to the end of the last coil (fig. 85). A casual examination reveals in the loose connective tissue oval shaped disks, which are not only located in the

tissues below the integumentum, but also between the intestinal loops, the folds of the digestive gland and in the vicinity of the uro-genital tract. On dissection, the bodies resemble the transparent concretions found in *Cyclostoma sulcatum* or *Leonia mamillare*. They are friable, mostly oval shaped, distinctly lamellated with a central core and tinged slightly yellow. As a rule, two dense layers are separated by a transparent, loose band of lamellae (figs. 86 and 92). In size they vary from 0.1 to 0.15 mm. in length and 0.05 to 0.1 mm. in diameter. A few of the bodies are distinctly crystalline, while the majority presents a waxy appearance. In dilute HCl or acetic acid or in Na_2CO_3 , they are insoluble. A 40 per cent. NaOH solution dissolves part of the bodies and leaves a shadow-like matrix. In dilute neutral red the outer layers stain faintly reddish. The histo-chemical behavior identifies these bodies as concretions similar but not entirely identical with those found in the deposits of other Cyclostomatidae. It appeared important to conduct a few qualitative chemical tests concerning the nature of the concretions. They are readily dissolved in acidified water. The solution gives a strongly positive reaction with Folin's uric acid reagent. A positive murexide reaction has also been secured, but the color reaction is not clean cut. In fact, a few tests with the small amount of material available suggests the predominance of xanthine bases. A series of quantitative tests will be made as soon as an adequate number of mollusks can be secured.

Unstained as well as stained preparations invariably failed to reveal an association of bacteria with the concretions. Furthermore, the oval concretions are not situated in cells or cell complexes. The origin and the cycle of the bodies may be followed on sections. It has been found advisable to cover the sections with a thin layer of collodium before passing them through the clearing and staining solutions. Without this precaution a number of concretions are washed out and are frequently deposited on areas of the sectioned tissues which have no connection with the original location. The findings, which are reported in this paragraph, have been made on sections stained with iron-hematoxylin or by the Giemsa method.

Clusters of from 10 to 30 concretions are located in the connective tissue surrounding small lymph or blood sinuses. Instead of being confined to a definite area in the region of the nephridium, these aggregations extend throughout the body of the mollusk. Concretions are found between the intestinal loops and the folds of the digestive gland (figs. 86 and 87). Practically every connective tissue zone in the vicinity of the lymph system is invaded by concretions. A superficial examination conveys the impression that the large oval bodies are located without any cellular boundaries directly in the spaces formed by the connective tissue. A more careful study under high power reveals however a number of cells with small or medium sized concretions. Based on a painstaking scrutiny of approximately 100 sections of two mollusks, the development of the concretions is believed to be intracellular. Two types of purinocytes have been found: (1) small cells with a dense nucleus, a small amount of alveolar cytoplasm which encloses a spherical body containing one or two "primary centres" (fig. 88), or a fairly large cell with an ellipsoidal nucleus containing numerous chromatin granules and a crystalline, lamellated and striated concretion (fig. 89), and (2) a large, oval shaped, typical concretion which carries a fine membrane and a compressed, flattened, rather deeply stained nucleus (fig. 91). In the latter type, no cytoplasm has been noted; in fact, the attached nucleus is the only remnant of the excretory cell. The majority of the large concretions even lack this cellular element. Up to a certain stage, the development of the purinocytes is practically the same as noted in the concretion deposits of *Cyclostoma elegans*. As soon as the products of excretion reach a certain size,

the cell is apparently superfluous, and its growth occurs independently of any cytoplasm in lymph spaces of the connective tissue. The varying thickness of the lamellae indicate that the development of the concretions does not take place continuously. Furthermore, the shape depends somewhat on their location; figures 87 and 92 leave no doubt that the concretions are modeled by the surrounding tissues. The specimens available for this study contained mostly fully developed concretions and have therefore not afforded an opportunity for a detailed inquiry into the growth and the histophysiological behavior of the purinocytes. One fact, however, is fully established, namely, the cytoplasm of the nephrocytic cells are never invaded by bacteria.

In the vicinity of the concretions, peculiar cells resembling the "néphrocytes de Cuénot" are quite common. They are elongated with a cytoplasm which regularly encloses several large and small deeply brownish bodies (fig. 90). Occasionally phagocytic cells singly or in groups have been observed. Regarding their activity nothing definite could be determined, at least no indications of any absorptive or destructive function on the concretions has been seen. However, no final conclusions can be drawn from the examination of two mollusks. Previous studies on other types of *Cyclostoma* have shown that the phagocytic activity is very low at certain periods of the year and even in different individual snails collected at the same time and in the same locality.

The nephridium of *Chondropoma dentatum* presents the same general structure and histophysiological activity as *Cyclostoma elegans*. The lamellae pierced by lymph lacunae and projecting into a spacious nephridial cavity carry one layer of excretory cells (fig. 87). The nephridial cells discharge large, clumpy yellow-greenish or small brownish, granular excreta.

In conclusion, it may be stated that a group of "Cyclostomatoid" mollusks has been found which possess purinocytes and concretions freely distributed throughout the connective tissue of the body. The purinocytes are never invaded by bacteria.

These findings establish for the first time the existence of concretion deposits in the representatives of the new family Annulariidae, which has recently been created by Henderson and Bartsch. This new family includes all the New World "Cyclostomoid" mollusks placed under the family name of Cyclostomatidae. The chief distinguishing character separating this group from all other operculate pulmonates is found in the radula, which fundamentally is uniform throughout the large number of species. The Old World Cyclostomatidae possess multicuspid rachidian, lateral, and inner marginal teeth, while the rachidian and lateral teeth of the Annulariidae are always unicuspid. Until Henderson and Bartsch had examined the radula of many species, authors hesitated to separate the American from the Old World forms, believing that the radular differences in American species showed a range of variation embracing the characteristics of the Old World group and those of the family Annulariidae. The demonstration of concretion deposits, either with or without symbiotes, throws an entirely

new light on this classification. It may be mere coincidence, but the large "Cyclostomatoid" mollusks from Japan and the Philippines showed no concretion deposits, while five of the six species collected in the Greater Antilles showed this organ. Superficial examination of the nephridia of the large Philippine species *Glossostyla validus* and *appendiculatus* indicates a different structure from that seen in the other species studied in this series. It is rather suggestive that the Cyclostomatidae of the islands of the Pacific Ocean differ in their purine metabolism from the species found in Europe, Africa, the Greater Antilles and possibly North and Central America. The presence or absence of concretion deposits originally thought to be characteristic for the genus *Cyclostoma*, has now assumed not only an importance in the classification, but also in the zoo-geographical distribution of the Cyclostomatidae. This aspect of the problem will receive careful attention in future studies.

BACTERIOLOGICAL STUDIES

AN ATTEMPT TO CULTIVATE THE INTRACELLULAR BACTERIA OF *CYCLOSTOMA ELEGANS*, *CYCLOSTOMA SULCATUM* AND *LEONIA MAMILLARE*

In the introduction it has been stated that the successful propagation of the intracellular bacteria on artificial mediums constitutes the only decisive argument, which permits of designating them as parasites or as "symbiotes." But it has been pointed out that in most cases the artificial cultivation experiments have so far met with unsurmountable difficulties, or the identification of the cultivated symbiotes has been unsuccessful. When, at the suggestion of Prof. J. Strohl, I considered the problem of cultivating the intracellular bacteria of the purinocytes of *Cyclostoma elegans*, I expected little or no difficulties, on account of the fact that Garnault and later Mercier had already reported successes in propagating the micro-organisms on culture mediums. The data detailed in subsequent paragraphs furnish ample proof that this assumption was wholly erroneous. Garnault, who had intended to test the pure cultures of the bacteria regarding their ability to destroy or to synthesize uric acid, writes as follows (p. 60): "Je crois être arrivé, par le procédé de Koch, à obtenir des cultures pures du bacille, mais les difficultés expérimentales qui s'offraient à moi pour arriver jusqu'au bout de mon programme m'ont déterminé à différer ces

recherches qui me semblent présenter un grand intérêt." More recently Mercier claims to have secured colonies of the bacteria of the "glande à concrétions" on various solid mediums, in particular coagulated blood of *Helix pomatia*. From a morphological and tinctorial point of view, the cultivated bacilli resembled those of the purinocytes. Mercier was perfectly right when he concluded that these characteristics were insufficient to establish an identification with any degree of certainty. In his opinion, it would be necessary to reproduce the infection in *Cyclostoma elegans* by the inoculation of pure cultures. Unfortunately, he was unable to secure specimens of these mollusks which were not already infected. Neither of the two investigators has made a careful biochemical study of the pure cultures, and no bacteriological or serological identification has been attempted. A detailed description of the experiments I have undertaken to cultivate the symbiotes of *Cyclostoma elegans*, *sulcatum* and *Leonia mamillare* is herewith given in order to indicate the procedure and the criteria, which should be chosen in the interpretation of the results. This course appears justified as zoologists may gain some insight into the many difficulties which may confront them in the investigation of similar problems.

When planning the technical execution of the bacteriological studies, it was fully appreciated that three points deserve special attention: (1) the preparation of the mollusks for the culturing of the concretion deposits; (2) the composition and the reaction of the culture mediums, and (3) the identification of the isolated bacteria.

(1) *The Preparation of the Mollusks for Culturing of the Concretion Deposits.*—The mollusks live in intimate contact with decaying vegetation and soil. A few preliminary tests established the presence of a variety of spore-bearing aerobes and anaerobes on the shell, in particular on the operculum and on the foot. In order to sterilize these parts, numerous experiments have been made with various antiseptics, but finally the following procedure has yielded very satisfactory results. The snails were scrubbed with warm water and liquid soap, rinsed in sterile tap water and then placed for 10 minutes in 3 per cent. liquor cresoli saponatus (temperature, 40-45 C.), carefully washed in sterile alkaline water (P_H 8.6) and then transferred for 15 minutes to an alcoholic solution of brilliant green and crystal violet (1 per cent. of each in 50 per cent. alcohol). This antiseptic solution has been devised by Browning and successfully used by Berwick and Meyer in their studies on the bacterial flora of abscesses in the human buccal cavity. The dyes have specific bacterioscopic properties for the gram-positive and gram-negative bacteria without exerting any harmful effect on delicate tissues. Furthermore, the alcoholic solution diffused readily through the crevices of the operculum and the shell; as a rule, the foot was deeply stained. Numerous control cultures in liquid and solid mediums proved the efficacy of the procedure; the contaminating flora was

reduced to a few spore-bearers; in fact, quite frequently sterile cultures were obtained. The dyes were washed off in 70 per cent. alcohol; the mollusks were dried by flaming them over a burner. The shell was broken with the aid of sterile forceps, while the mollusk was held between two fingers covered by sterile rubber cots. The aseptic removal of the operculum offered some difficulty, until it was found advantageous to cut off a portion of the foot by means of sterile scissors. The naked snails were pinned to a layer of sterile paraffine wax, which had been poured in sterile Petri dishes. At this stage the specimens were ready for culturing. In case the entire concretion deposits were removed, it was found practical to cover the foot and the upper end of the mollusk with cotton soaked in tincture of iodine.

With the aid of a dissecting binocular lens or microscope, the removal of small fragments of the concretion deposits was controlled. Sterile capillary pipetes were excellently suited for the dissection and aspiration of the specimens to be cultured. Utmost care was exercised to avoid injury of the intestinal loops passing through the concretion deposits. In the majority of instances the specimens, which were plated or enriched, were taken below the integument in the proximity of the nephridium. Cultures of the intestinal content were obtained as follows: The intestinal loop passing through the concretion deposit was dissected from its anterior and posterior connections; it was carefully stripped of the adherent connective tissue and rinsed in saline. A fine pipet was then inserted, and one or several fecal balls removed to sterile slides, on which they were emulsified in salt solution with a small amount of sand. Frequent sterility tests of the surface of the integument, etc., in liquid mediums were made during the culturing of a series of mollusks. As already stated, these cultures remained sterile in the majority of instances.

(2) *Composition and Reaction of the Mediums.*—The subject of the cultural isolation of symbiotes found in invertebrates has been considered in a few publications. Considerable difficulties have been encountered by a number of workers, but an analysis of the cultural requirements of the bacteria, molds or yeasts thus far isolated have not been made. In devising artificial mediums suitable for the isolation of the symbiotes demonstrated in the purinocytes of *Cyclostoma elegans*, three possibilities suggested themselves. (a) The symbiotes are highly parasitic; their metabolism is highly specialized, cultivation requires, therefore, special amino-acids and probably growth promoting vitamins. (b) The organisms are soil bacteria, or they are similar to the microbes found in the "bacteroids," of the roots of leguminous plants, and therefore they may develop poorly or not at all on culture mediums containing large amounts of organic material. (c) The intracellular micro-organisms are representatives of the ubiquitous flora of decaying vegetation, water, etc.; cultivation on ordinary artificial mediums is then readily accomplished.

This is not the place to discuss the various conceptions regarding the composition of culture mediums necessary for the artificial cultivation of highly parasitic micro-organisms. Although considerable progress has been made in recent years,* it remains a fact that various intracellular parasites have not as yet been cultivated. Among the factors which deserve attention in an attempt to cultivate parasitic bacteria, three are of prime importance: first, the concentration of the hydrogen-ions, or the reaction; second, the concentration of the amino-acids; and third, the presence of growth stimulating vitamins. In the following paragraphs a brief resumé will be given of the procedures

* Wolbach, Pinkerton and Schlesinger have cultivated the organism of Rocky Mountain spotted fever and typhus in tissue cultures.

which were chosen to adapt a number of culture mediums to the condition supposed to be necessary for the cultivation of the symbiotes of *Cyclostoma elegans*.

Reaction: The studies of a large number of workers have clearly demonstrated the influence of the hydrogen-ion concentration on bacterial activity in artificial culture mediums. For various micro-organisms the optimal initial P_H , the optimal zones of growth, the optimal zones for general or special metabolism, the optimal zones for preservation, etc., have been determined. These studies have been materially facilitated by the introduction of indicators with a characteristic P_H zone. In the tests to be reported the indicators recommended by Clark have been used. It has been assumed that the optimal reaction for the growth of the symbiotes is near the reaction of the body fluids of the mollusks. A number of determinations of the P_H of the lymph and tissues of *Cyclostoma* have been made. The spotting method of Felton was employed with success. In case large amounts of tissue fluids were available, dilutions of 1:5 were made in capillary tubes, and the dilutions were compared after the addition of the indicators with colored buffer solutions. It is impossible to secure sufficient lymph to make electrometric determinations. The average reaction of the lymph and body fluids of 25 *Cyclostoma elegans* corresponded to a P_H of 7.4. In some mollusks, the P_H was as high as 7.6 or as low as 7.2; the boiled extract of 10 *Cyclostomas* had a P_H of 8.4. The P_H of the lymph of 6 *Cyclostoma sulcatum* and that of 5 *Leonia mamillare* was approximately 7.3 to 7.4. These results are comparable with the data reported by Quagliariello (p. 641), who determined the hydrogen-ion concentration of the blood of a number of mollusks by means of the electrometric method. This worker found the body fluid of mollusks to possess a slightly alkaline reaction approximately of the same degree as the blood of higher vertebrates. According to his studies, the buffer value or the ability of the blood to fix acids or bases stood in close relationship to the concentration of the proteins. The decrease of the hydrogen-ion concentration of the blood on boiling may be attributed to the presence of bicarbonates, an explanation already given by Gautrelet for a similar observation made on the blood of *Helix pomatia*. Based on these data, all culture mediums used have been adjusted to a reaction of P_H 7.3 to 7.5.

Concentration of Amino-Acids.—From the work of Bainbridge and the studies of Sperry and Rettger and Rettger, Berman and Sturges it is evident that the amino-acids and other nitrogenous substances which readily give up their nitrogen as a result of bacterial action, are particularly responsible for the food value of culture mediums. Even strongly proteolytic bacteria are unable to attack native proteins. Indeed, some evidence suggests that certain amino-acids, single or in mixtures belong to the basic food requirements of certain parasitic bacteria. It has been customary to secure these substances by extracting the organ of the host, in which the parasites are found. In this connection, it is recalled that Blochmann (p. 36), who attempted the cultivation of the bacteroids in the body of cockroaches, prepared a cockroach-infusion medium, while Zirpolo (350) and Pierantoni in culturing the light organs of *Sepiolo* employed a special "*Sepiolo*" broth. Mercier (1913, p. 18) reports that he obtained the growth of a gram-negative bacillus in culturing the concretion deposits of *Cyclostoma elegans* on coagulated blood of *Helix pomatia*. These and similar statements prompted the use of culture mediums prepared from mollusks, in particular *Cyclostoma* and *Helix*. Preliminary tests indicated that the addition of several types of peptone was superfluous; in fact, it was noted that the growth was more characteristic on the medium without peptone. It

was, however, found that the addition of glucose greatly enhanced the growth of the bacteria. Accordingly, 0.5 to 1% glucose was added to most of the special mediums.

Growth Stimulating Substances.—Thjotta and Avery, who review the comparatively recent work on vitamins in bacterial nutrition, found that "two distinct and separable substances, both of which are present in the blood, may play an important rôle in the cultivation of a number of bacteria." Cole and Lloyd found that the substance of importance in inducing initial growth was probably a derivative of the red blood cells and was shown to be readily absorbable by colloidal substances, such as agar and gelatin. The second substance present in animal and plant tissues, was relatively nonabsorbable, and was thought to stimulate luxuriant secondary growth. The first substance was considered to be of the same nature as vitamins or hormones, because of the ease with which it was absorbed. Huntoon, utilizing these principles of Cole and Lloyd has described simple methods for preparing these vitamin mediums. Instead of blood, beef heart or steak was employed with the idea that these tissues would provide sufficient of the growth accessory substances, especially when fortified with a whole egg. Mediums of this type have been found very useful in the work with the symbiotes of *Cyclostoma elegans*. Beef hearts have been used as a meat base; in later experiments, however, *Helix pomatia* and *Cyclostoma* mollusks have been employed. The mode of preparation of these mediums is described here in order to encourage their use by later workers on the same subject:

Two hundred and fifty grams of fresh, chopped beef heart or mollusks, one whole egg and 500 c.c. of tap water are placed in a double boiler over a free flame and the temperature maintained at 60 C., with constant stirring, for 5 minutes. Ten grams of flaked agar and, if desired, 5 gm. of peptone are now added, and the temperature raised until the mixture assumes a brownish color. The medium is then made slightly alkaline, using a 10% solution of sodium carbonate. It is then placed in a flask and heated at 100 C. in the steam sterilizer for one hour. The clot is then separated from the sides of the receptacle, and it is placed in the sterilizer for another hour. It is then cleared by straining through glass wool. After filtration, the reaction is brought to P_H 7.2. It is then distributed in flasks or tubes and sterilized by fractional sterilization at 100 C. flowing steam.

In a number of experiments the nutritive value of the medium has been enhanced by the addition of 10% defibrinated rabbit blood or filtered *Helix pomatia* or *Cyclostoma* extract. The latter was prepared in accordance with the principles given by Krontowsky and Rumianzew in devising a medium for tissue cultures of *Lumbricidae*.

About 10 to 20 large cyclostomas, carefully sterilized according to methods already described were ground with sand in a mortar. An amount of Ringer's solution equal to the weight of the mollusks without sand was added and the slimy mass shaken for 10 minutes. It was then centrifugalized at low speed. The supernatant fluid was removed, mixed with the hemolymph of about 20 to 40 mollusks and passed through a diatomaceous filter. In a number of instances, it was impossible to filter the stringy material; sterilization was then accomplished by the addition of chloroform and preservation in the ice chest for 4 weeks or by sterilization in the flowing steam by 100 C. for 30 minutes. As the total yield of these extracts was usually small, only 1 to 2% were added to the agar, just before pouring.

The various other mediums employed in the course of this study were prepared according to standard formulas and will be mentioned when detailing the experimental records of the different series. Anaerobiasis in liquid mediums was secured by stratification with yellow vaseline. The efficacy of this method has been experimentally proved by a number of bacteriologists in particular by Olitsky and Gates. For plate cultures, the procedure of Marino as modified by Krumwiede and Pratt,* has given excellent anaerobic cultures. Within recent years a number of investigators have advocated a reduced oxygen tension atmosphere as favorable for the growth of certain parasitic bacteria (*gonococcus*, *B. abortus bovis*, etc.). This reduction has been effected in various ways: by heating the air in the culture tube and closing tightly with a rubber stopper, by partial exhaustion of the air; by exposure to a CO₂ atmosphere; by the use of a bacterial culture with oxygen reducing properties, such as *B. Subtilis*. From time to time the first and the last named procedure have been tested, but the results have in no way encouraged their use; in fact, it was evident that other factors than a reduced oxygen tension were responsible for the numerous sterile cultures secured from the concretion deposits of *Cyclostoma* studied during hibernation.

3. *The Identification of the Isolated Bacteria.*—The routine procedure adopted in culturing a large series of concretion deposits consisted of smearing small fragments of the tissues on the surface of several plates. Appropriate dilutions were secured by spreading the material with a bent platinum wire. Occasionally portions of the specimens were carefully emulsified in some liquid medium and the dilutions used for the seeding of the plates. In several series, pieces of the organs were incubated at 22 C. in a liquid medium for from 12 to 24 hours before plating. Invariably the presence of the symbiotic bacteria was controlled by the examination of microscopic smears. All cultures in liquid or solid mediums were incubated at 22 C. for at least 10 days. As soon as the liquid cultures revealed some turbidity, or latest on the 10th day, seedings were made on a variety of solid mediums.

The plate cultures were daily examined, and at least three colonies of the various types which had developed transferred to slanted solid mediums and microscopically examined. It was soon realized that the concretion deposits of the *Cyclostomatidae* may harbor bacteria which frequently produce mutations. This fact made the examination of the plates a tedious and laborious procedure. Indeed, in a series of very careful analyses during which two-thirds of the colonies appearing on one plate were studied morphologically and biochemically, it was finally found that the bacteria with different colony characteristics all belonged to one and the same species. As a routine procedure all strains after adequate purification were tested on the following mediums: gelatine, glucose, lactose, maltose, saccharose and mannite Andrade's indicator broth, Witte's peptone solution for indol (Ehrlich's reagent), phenol red broth, urine with phenol red, uric acid-phenol-red-phosphate solution for ammonia production, bromcresol-purple-milk, neutralred-agar, urea-agar according to Söhngen and rabbit blood-beef extract agar. It is self explanatory that only the gram-negative bacteria were studied in detail. The analyses of 526 single cultures lead to the classification of the strains into 4 large groups, which will be discussed in subsequent paragraphs.

* The inoculated agar is poured in the upper half of a Petri dish and then covered with the inverted lower half; the open crack between the two halves of the dish are sealed with paraffin wax.

CULTURES PREPARED FROM THE CONCRETION DEPOSITS
OF CYCLOSTOMA ELEGANS

The examinations of *Cyclostoma* were begun in November. The mollusks secured for the first six series were hibernating and were kept in dry soil at an even temperature of the laboratory. In later series, active field specimens were studied. A total of 124 *Cyclostomas* has been cultured. The experiments are herewith presented in detail:

Series 1.—This series was undertaken to control the procedure of sterilizing the shell of the mollusks and to practice the method of dissecting them under aseptic conditions. A detailed study was made of the intestinal flora of 3 snails of different sizes. Simple mediums with the addition of horse serum and sheep blood were employed.

It was decidedly startling to find the majority of the plates either free from any bacterial growth whatsoever or presenting after prolonged incubation one or two colonies of gram-negative bacilli, which in many ways corresponded to those isolated from the intestinal tract. On the sterile plates, the symbiotes which had been seeded were readily demonstrable in microscopic smears prepared from the surface of the medium. Among the bacteria isolated in the plates of this series the pigmented types, which were readily recognized as variants of the *Bacterium fluorescens* and *Bacterium herbicola*, predominated; however their significance was not understood. In fact, the same types were demonstrated in the intestinal tube of the mollusks, and it was suspected that in the course of the removal of the concretions a contamination with fecal material took place. On account of these results, it was concluded that the culture mediums were inadequate and another series was prepared.

Series 2.—Five concretion deposits were seeded on vitamine-blood agar and on urea—(2%) uric acid (0.5%) and urine—(10%) agar. The latter mediums were prepared by adding the sterile urea-solution or the uric-acid (sterilized in a dry state at 160 C.) in powder form or the aseptically collected urine to the slightly alkaline vitamine-agar. Aerobic as well as anaerobic plates were prepared. A small fragment of the concretion material was also transferred to beef heart enrichment tubes.

The results of series 2 differed very little from those recorded for series 1. Diffuse growth was found on the plates smeared with specimens removed from a mollusk, in which contamination with intestinal content was evident. The absence of visible growth on the urea, uric acid and urine plates must be attributed to the fact that probably little or no concretion material was transferred from the blood plates, on which minute fragments had been spread. No growth took place on the anaerobic blood plates or in the beef heart mince. The isolated bacteria were gram-negative bacilli and cocci similar to those found in series 1.

Mercier (p. 18) conveys the impression that the gram-negative bacilli developed particularly well on coagulated blood of *Helix pomatia*. This claim has been investigated. For technical reasons, it is impossible to secure enough *Cyclostoma* lymph to cultivate the concretion deposits on the coagulated substratum. Several purinocytes have, therefore been teased in fresh, sterile lymph and hanging drop preparation of this material have been incubated in an aerobic and anaerobic Hansen's single cell cultivation chamber (Itano and

Neill). Isolated bacteria have been examined under the microscope for a week, but no dividing forms, multiplication or even disintegration have been seen. The drops have finally been smeared on mollusks extract agar; a few colonies of gram-negative bacilli belonging to the "fluorescens group" or "alkaligines" group developed.

Series 3 and 4.—The findings of series 2 suggested the culturing of a large series of mollusks on vitamine-blood agar; in employing one medium, it was expected that individual differences in the bacterial flora of the concretion deposits would be more readily detected than on a variety of mediums of different biologic value. Furthermore, Cyclostomas from three regions in Switzerland (Canton Baselland, Tessin and Geneva) have been cultured. A total of 37 hibernating mollusks have been studied.

Twenty-three Cyclostomas from Liestal, 10 from Geneva and 4 from Mendrisio (Tessin) have been cultured. In the course of the sterilization of the shell, it was noted that the mollusks can be roughly divided into 2 groups: "floaters" and "sinkers." Under the term "floater" are understood Cyclostomas, which float in the antiseptic solution; as a rule, the mollusks are retracted in the shell rather dry, and a considerable air space is found between the fleshy parts and the shell. On the other hand, the "sinkers" fill the entire inside of the shell; the connective tissue surrounding the intestines, etc., is moist. These animals usually exhibit definite signs of life. The same differences were exhibited by female as well as male Cyclostomas. In analyzing the cultures of the concretion deposits of the Cyclostomas collected at Liestal, it was noted that 8 or nearly one-third of the mollusks presented organs from which no bacteria could be cultivated on blood-agar plates. In 7 instances, the animals were definite "floaters" with very dry concretion deposits, while in others, they were very fine and only visible as small bands. On three plates either alone or predominantly gram-negative cocci were demonstrated; on the remaining plate series, gram-negative rods developed in colonies of varying morphologic appearance. Careful identification placed the rods of 7 plates into the fluorescens group, while a mixture of bacteria of the alkaligines, herbicola and coli groups were recorded on the remaining 5 plates. In this connection, it must be emphasized that the number of colonies visible on the plates was small in proportion to the seedings with concretion material. Indeed the microscopic demonstration of the symbiotes between the colonies of the actively growing gram-negative rods was again readily accomplished. Four Cyclostomas furnished plates with one type of bacteria, *B. fluorescens liquefaciens* and non-liquefaciens, in two instances the mollusks were "floaters," while in the two others considerable moisture was present in the deposits. Although *B. fluorescens* predominated in the bacterial flora of this series, representatives of the coli and alkaligines group attracted attention. A few colonies of the herbicola group appeared on the plates of three different mollusks.

The cultural findings on the Cyclostomas collected in the Jura méridional near Fort de l'Ecluse, Geneva, differed from those just described by the predominance of bacteria belonging to the colon group. On some plates only few, while on others innumerable colonies of the same type made their appearance. Fluorescens, alkaligines and herbicola representatives were found on one plate each. The first two named covered the surface of the mediums in a thick layer. Biochemically and morphologically the gram-negative bacteria isolated from the Geneva specimens differed only in minor details from those cultured from the Liestal Cyclostomas.

The 4 specimens of *Cyclostoma* obtained from Mendrisio furnished little information of value; the cultures of two remained sterile, while one developed colonies of gram-negative cocci. The mollusk yielding a pure growth of fluorescens types was a "floater." Various types of bacteria, mesentericus, coli, fluorescens, herbicola, proteus and gram-negative cocci were found in the intestinal canal. Careful attention was paid to the cultural characteristics of the fluorescens types isolated from the intestinal canal of No. 47 and the corresponding type found in the concretions deposits. Ten colonies of each were studied, but aside from the characteristic pigment production, differences in the ability to liquefy gelatine, to ferment glucose and to produce ammonia were found. Subsequent serological studies failed to establish an identity of the strains.

The results of series 3 and 4 differed in no respect from those reported in series 1 and 2. It was impossible to designate with certainty the representatives of the 4 groups of gram-negative bacteria as the microscopically demonstrable symbiotes, notwithstanding the fact that great similarity in size and arrangement, as well as the frequent occurrence, threw great suspicion on the fluorescens group.

At this stage of the investigation, it was realized that the mollusks used in the study were all hibernating forms, mostly kept under abnormal conditions. The data available indicated that the dry forms yielded mostly sterile cultures. It was reasoned that the cellular metabolism of the purinocytes was at a very low stage. Furthermore, the microscopic examination (Nyfeldt's and Kayser's test) indicated that most of the intracellular symbiotes stained poorly and were either dead or at least injured in their vitality. It was concluded that hibernating *Cyclostomas* were unsuited for the solution of the problem. The subsequent studies of large series (7-11) have with a few exceptions been made with actively living mollusks which were either recently collected in the field or kept in captivity under optimum conditions of food and moisture. It was naturally thought that this factor was not the only one responsible for the failure in culturing of what was considered the symbiotes. Numerous objections could be raised against the use of mediums prepared from bullocks heart. Extractives and amino-acids obtained from animals biologically more closely related to the host of the symbiotic bacteria offered theoretically some possibilities. Furthermore, soil bacteria as symbiotes were considered on account of the fact that some of the intracellular micro-organisms resembled fragments of actinomycetes. These ideas have been tested in series 5 and 6.

Series 5.—The following culture mediums have been tested in this series: *Helix pomatia*-vitamine-agar with and without glucose and peptone, Krainsky's dextrose agar (dextrose 10 gm; K_2HPO_4 , 0.5 gm. agar, 15 gm.; distilled water 1,000 c.c.); egg-albumin agar (dextrose, 10 gm.; K_2PO_4 , 0.5 gm.; $MgSO_4$, 0.2 gm.; $Fe_2(SO_4)_3$, trace; egg-albumin, 0.15 gm.; agar 15 gm.; distilled water 1,000 c.c.); calcium malate agar (calcium malate, 10 gm.; NH_4Cl , 0.5 gm.; K_2HPO_4 , 0.5 gm.; glycerine, 10 gm.; agar, 15 gm.; distilled water 1,000 c.c.; reaction adjusted by use of NaOH to P_H 7.4); soil extract agar with and without glucose; Beijerinck's enrichment medium for soil anaerobes (Na_2HPO_4 0.05 gm., ammonium sulphate 0.05 gm.; soluble starch, 1 gm.; calcium carbonate, 0.5; distilled water, 100 c.c.); beef liver peptone agar. The plates were heavily smeared with concretion material of *Cyclostomas* from 3 different localities.

The data clearly demonstrate the negative result of this series. One mollusk contained bacteria in the concretion deposits, which were capable of development as colonies. The organism was a pure culture of *B. fluorescens*. One colony on another plate was identified as a representative of the alkaligines group.

Series 6.—Two *Cyclostomas*, which had been kept on sterile, moist soil were cultured. The mediums were kept under aerobic and anaerobic conditions. With the exception of the hay-infusion agar, every solid medium was enforced by unheated *Cyclostoma* or *Helix pomatia* lymph or extract. The liquid mediums were sterilized by filtration.

The expectations which had been placed on the special culture mediums were not realized. In fact, additional experiments which were made with *Cyclostoma* lymph and not recorded in the tables, gave negative results. In six cultures, purinocytes were suspended in *Cyclostoma* lymph on sterile cover-glasses in moist chamber preparations. Several fields showing isolated bacteria or even small clusters were controlled daily under the microscope, but no signs of multiplication were detected. The culturing of *Cyclostoma* was interrupted until active mollusks directly collected from the forests were available.

Series 7.—Six active *Cyclostomas* collected at Liestal were carefully dissected. The concretion deposits were smeared on *Helix-pomatia-vitamine-agar* enforced by the addition of glucose and *Cyclostoma* extract, and on *vitamine beef-agar*.

Before discussing the bacteriological findings given in table 7, it appears advisable to record the appearance of the dissected mollusks. In contrast to the hibernating forms, it was noted that most of the snails expelled considerable fluid when closing the shell with the operculum. This phenomenon found its explanation in a marked oedematous-like, succulent condition of the foot and body tissues in general. Occasionally the peri-intestinal connective tissue exhibited a jelly-like, glassy, structure, which was studded with concretions of varying sizes. Lymph material was freely oozing from the tissues after the removal of the integument.

As a rule, either all or at least the majority of the plates seeded with the concretion deposits from these mollusks revealed an extensive growth of gram-negative bacilli. In fact, only one set of plates (*Cyclostoma* 59) gave in addition to the rods, colonies of cocci and yeasts. Size and appearance of the colonies was generally greater and more typical on the *vitamine-beef-agar*. Ammonium-magnesium-phosphate crystals were seen in most of the plates on the 5th day of incubation at 22 C. The flora was strongly ammonia producing. Identification places the various colonies in three groups: the fluorescens, alkaligenes and the *B. coli* group. The "alkaligenes" and *coli* types were relatively rare.

In this series, duplicate sets of the mediums were inoculated with the intestinal content of every *Cyclostoma*. In contrast to the plates similarly prepared in series 1, 3 and 4, the numerous colonies and the variety of bacteria which were recognized in the course of the microscopic examination, indicated a prolific flora in the digestive tube of a feeding and active mollusk. Gram-negative cocci, as a rule, represented one half of the colonies, while gram-negative rods, mainly colon types, made up the other half. Although it was technically impossible to examine all the colonies, it was by comparison evident that the concretion deposits harbored mainly fluorescens types, rarely cocci and colon types. This observation was furthermore strengthened by the microscopic appearance of the collective smears prepared from the heavily seeded "smear plates"* of the concretion material and the intestinal content. In the former gram-negative bacilli of varying length and size, while in the latter, a mixture of gram-negative and gram-positive cocci and rods, were seen.

* In order to secure plates with isolated colonies, it was necessary to spread the original specimen of intestinal content or concretion deposit over the surface of one plate. From this so-called "smear plate" transplants were made with a bent wire to the various other mediums and plates.

Series 8.—About the same time as the plates of series 7 were studied, a new shipment of *Cyclostoma* was received from Marseilles. Four of the largest specimens were cultured on *Helix-pomatia-vitamine-agar* with and without glucose. Three mollusks from Liestal were used as controls. Instead of using a medium with *Cyclostoma* extract, which did not furnish any better growth or colonies different from those found on the other mediums, glycerine-potato agar was employed as a "smear plate." The concretion deposits as well as the intestinal canal were cultured.

The data indicated that the finding on 4 *Cyclostomas* from Marseilles did not differ from those made on the same species of snail from Liestal or Geneva. Colon types were primarily isolated and identified, but fluorescens types were equally numerous. In one instance, a pure culture of *B. coli* was obtained. It may be mere coincidence, but no cocci or gram-positive rods were found on the plates prepared from the concretion deposits, while the intestinal content contained these types in considerable numbers. These and similar observations indicate that the concretion deposits have, as a rule, a selective affinity for the gram-negative bacteria of the intestinal flora. The constant development of numerous colonies from a relatively small fragment of tissue rather forcibly suggested some relationship between the cultivated gram-negative bacilli and the symbiotes. Such a relationship was considered even more probable, since it was demonstrated that two of the four bacterial groups were capable of using pure uric acid as the sole nitrogen source for their metabolism. It was considered advisable to combine in subsequent series of cultures direct plating with elective enrichment of the concretion material in uric-acid-phosphate solutions. These experiments are detailed in series 9, 10 and 11.

Series 9.—Vitamine-beef agar, urea-agar (5 c.c. of a 40 per cent. aqueous solution of urea sterilized at one half atmosphere for 30 minutes added to 100 c.c. of melted and cooled vitamine-beef agar) were used as solid mediums. An enrichment-medium the uric acid phosphate solution of Stapp* with a P_H of 7.4 was employed.

Four *Cyclostomas* each, originally secured from Liestal and Geneva, had been kept since the beginning of February for a period of 80 days in damp flower-pots with ivy at the temperature (18-20° C.) of a hot house at the Botanical Garden in Zürich.† For 3 days they remained in sterile glass jars, in sterile soil, which was moistened with sterile tapwater. It was assumed that this treatment would stimulate the general metabolism, the turgor of the tissues and a dissemination of the intestinal bacteria by the lymph. As controls served four *Cyclostomas* recently collected from the forest colony at Liestal and several specimens from Liestal, Geneva and Tessin, which had been kept on dry sand for at least 5 to 6 months.

An analysis of series 9 reveals a number of interesting facts: 1. The treatment with sterile water increased the hemolymph of the mollusks. 2. The nephridiums of the 8 *Cyclostomas* kept for 80 days in the hot house are very large. 3. The concretion deposits are, as a rule, small; it is not unlikely that

* A. Uric acid.....	05	B. Mineral solution	
Na ₂ HPO ₄	3.0	KH ₂ PO ₄	1.0
Mineral solution B.....	50.0	CaCl ₂	0.1
Distilled water.....	450.0	Mg SO ₄ + 7H ₂ O	0.3
Phenol red to color.....	NaCl	0.1
		Fe ₂ Cl ₃	0.01
		H ₂ O	1000.0

† I am indebted to Prof. Dr. H. Schinz for permitting me to keep the snails in the Botanical Garden.

the metabolism is considerably activated by the high temperature, causing a visible diminution of the concretions. 4. The lymph from the posterior aorta of the treated mollusks is invariably sterile. 5. The concretion deposits contain innumerable viable bacteria; gram-negative bacilli predominate, but cocci are also found. 6. The frequent demonstration of pigmented colonies consisting of gram-negative bacilli of the "herbicola group" is striking. 7. The absence of the colon types in the concretion deposits of the incubated mollusks and the predominance of these types on the field specimens from Liestal confirm previous incomplete observations. 8. Growth in the uric acid medium is indicated by ammonia production, which increases the H-ion concentration of the solution from P_H 7.2 to P_H 8.4 to 8.8; subcultures give, as a rule, a pure growth of gram-negative bacilli belonging to the fluorescens group. 9. On the vitamine agar pigmented colonies mixed with spreaders (fluorescens types), while on the urea-agar mainly colonies of the "alkaligenes group," attract attention. 10. The intestinal flora exhibits a very rich flora of gram-negative and positive cocci and rods; pigmented colonies of cocci and numerous spore-bearing rods of the mycoides and mesentericus groups are rare. 11. As a whole, the flora of the concretion deposits differs from that of the intestinal content by its predominance of gram-negative bacilli. 12. The direct surface seedings from the concretions of the hibernating mollusks furnishes sterile cultures. The enrichment cultures give rise to "fluorescens" colonies. Relatively few colonies have developed on the mediums inoculated with the intestinal contents of these mollusks.

The results of this series confirm and enhance previous conclusions, namely, that the concretion deposits of active mollusks harbor micro-organisms common to the intestinal flora. Cyclostoma, which are held in a moist environment, show large numbers of viable bacteria in the peri-intestinal connective tissues, although no organisms have been demonstrated in the hemolymph of the posterior aorta. The flora consists mainly of gram-negative bacilli, which form alkaline-split products; some possess the property to decompose uric acid. The latter types can be enriched in a medium containing uric acid as the sole nitrogen source. The contrast in the flora of the concretion deposits of active and hibernating Cyclostomas as demonstrated in this series is striking and fully explains the variable or negative results secured in series 1-6. Indeed these findings lend further weight to the belief that the majority of the microscopically demonstrable symbiotes are either dead or dormant or seriously injured in their vitality. The encouraging outcome of the tests reported here as series 9 were responsible for series 10 and 11.

Series 10 and 11.—These two series can be discussed together. The procedures of culturing and the mediums used were practically the same as in series 9. The Cyclostomas recently collected at Liestal were kept in moist soil with an abundance of green food as salad, carrots, clover, etc. It was of interest to know whether or not a change of flora took place in the mollusks, which were kept in captivity on nonsterile soil. The mollusks from Nice and Grenoble were received in small tin containers; they were immediately transferred to sterile dishes with sterile damp soil. Precautions were taken to avoid cross contamination between the snails obtained from Nice and those from Grenoble. Considerable interest was attached to the solution of the question: "Is the flora of the concretion deposits of the Cyclostomas from Grenoble similar or identical to that of the snails from Nice?" In this connection, it is recalled that for example the flora in the concretion deposits of mollusks collected in Geneva (series 4) consisted mainly of colon types, while simultaneous cultures of Cyclostomas from Liestal yielded fluorescens types.

The results on 39 mollusks are briefly as follows:

The flora of the concretion deposits of *Cyclostomas* from Liestal remains the same in captivity, for example *fluorescens* types are just as predominant in series 10 as they are in series 7. The age of the mollusks has apparently no influence; the snails of series 10 belong to the large forms, those of series 8 to the "forma minor." In both series animals which are actively moving around have been cultured. No differences are noted in the flora of the intestinal tract. However, two observations seem to confirm the results of previous series. *Cyclostoma* 90 and 95 yield few colonies on the ordinary medium; a spreading growth is found on the uric acid plate of No. 90. A definite relationship between the size of the concretion deposits and the number of viable bacteria in active mollusks is evident; both No. 90 and 95 present fine band like concretions in a jelly-like infiltrated connective tissue. Removal of the specimens offered no difficulties; in fact, mainly concretion cells and considerable lymph have been aspirated with the pipets. The plates have been thickly seeded, and yet the number of colonies which developed is small. Scanty and delayed growth has been noted on the plates prepared from *Cyclostoma* 93; the mollusk possessed extensive cement-like, dry concretions. Previous experience has shown that, as a rule, few viable organisms develop on the cultures from these specimens. From these observations it is concluded that viable bacteria other than representatives of the *fluorescens* group are rare in the large but very dry, and in the very small but oedematous-like concretion, deposits. In the former condition, the microscopic examination reveals numerous free, but mainly silver positive, bacteria, which are probably dead, while in the latter few clusters of silver-negative rods are seen. As no other bacterial, particularly typical intestinal types, are demonstrable, it is not unlikely that the clusters of gram-negative rods are the actual symbiotes which develop into single colonies and as such mask the actual number of viable bacteria. Such an explanation is supported by the fact that enrichment in uric acid medium gives abundant growth, while the procedure applied to the large, but, dry, concretions gives negative results. The intestinal flora of the mollusks in series 10 is characterized by a predominance of colon types with gram-negative cocci and a scarcity of representatives of the *herbicolo* group.

The conclusions from previous experiments justify the assumption that a uric acid medium is to be preferred in place of a substratum rich in nonspecific peptones and amino acids. As direct plating on special mediums failed to furnish additional information, the last series 11 was prepared by using an enrichment medium. The enrichment cultures were plated after 12 and 144 hours' incubation at 22 C. In two instances, parallel experiments on solid mediums and in enrichment cultures were carried out. It was found that the main types of the flora in the concretion deposits were present in the same proportion after 12 hours' incubation as on the direct plates. After 144 hours, however, the gram-negative rods belonging to the *fluorescens* and *alkaligenes* group were the predominant types.

The results of this series contributes several valuable facts. The observations on hibernating mollusks are confirmed by the findings on *Cyclostoma* 97 and 98; growth is scanty or absent, even in the enrichment tubes. The differences in the bacterial flora of the concretion deposits of *Cyclostomas* from Grenoble and Nice are striking. The cultures of the latter mollusks develop gram-negative cocci and *herbicola* types. It is quite significant that a pure growth of gram-negative cocci has been obtained from the concretion of three large and active mollusks. Only 5 to 12 snails harbor *fluorescens* types; these gram-negative bacilli are in the minority. A superficial comparison of the plates prepared

with specimens from Grenoble and those of Nice demonstrate further points of difference. An abundance of crystals or distinct hemolysis on the blood plates of the Grenoble material contracts sharply with the Nice plates, which are dotted with small raised and pigmented colonies. In a number of smear preparations made with the Nice specimens, gram-negative cocci have been demonstrated. The gram-negative bacteria which formed nonpigmented colonies on the plates of the Grenoble material behave like *fluorescens* types without pigment. It is not unlikely that the colonies on the various plates are mutants of the same genus. The absence of colon types was striking. The Liestal specimens, which served as controls yield cultures which are identical to those reported in series 9.

CULTURES PREPARED FROM THE CONCRETION DEPOSITS OF *CYCLOSTOMA SULCATUM*

In the light of the bacterioscopic findings made on the purinocytes of *Cyclostoma sulcatum* obtained from Nîmes and Marseilles, it appeared imperative to prepare a series of cultures with the concretion deposits of this species. Although the snails available were slightly larger than the forms of *Cyclostoma elegans* employed in this study, it must be recalled that the concretion deposits are shieldlike and relatively small. However, the deposits are rarely intimately connected with the intestinal loops and can be readily removed in toto. Furthermore, the sinuses surrounding the concretions are frequently not engorged with lymph, a condition which enables the operator to secure specimens with very little contamination by the intestinal flora.

The direct cultures of 6 *Cyclostoma sulcatum* from Nîmes furnished sterile plates in 4, while those of the mollusks from Marseilles supplied in 5 of 6 cultural attempts not only sterile plates, but also sterile enrichment cultures. The bacteria of the purinocytes are readily demonstrated in the smears made from the plates or tubes, which exhibit no visible growth. The gram-negative organisms, which grow in isolated colonies or by enrichment belong to the *fluorescens* group. Cocci and diphtheroids, possibly contaminations from the hands of the operator, have developed on two plates. In comparison with the findings reported for the cultures made with *Cyclostoma elegans*, the results are interesting from two points of view. (a) The bacteria demonstrable in the purinocytes of *Cyclostoma sulcatum* are not cultivated either direct or by enrichment in mediums, which give as a rule good growth of several definite types of gram-negative bacilli. (b) Bacteria belonging to the intestinal flora are either absent in the direct plates made from the concretion deposits, or the *fluorescens* types are the only representatives. The cultures prepared from the intestinal content produced colonies of the same bacterial types as noted in *Cyclostoma elegans*. This outcome of the cultural studied on *Cyclostoma sulcatum* is rather surprising, as the bacterioscopic methods ("Cyanochin" and silver-nitrate test) have indicated that the majority of the symbiotes are viable. Furthermore, it is impossible to accept the *fluorescens* types as the symbiotes; the number of the colonies is insignificant, and morphologically the cultivated rods present no similarity, even when making proper allowance for the numerous variations common to the group. It must, therefore, be concluded that the intracellular symbiotes of *Cyclostoma sulcatum* have not been cultivated.

CULTURES PREPARED FROM THE CONCRETION DEPOSITS OF *LEONIA MAMILLARE*

A series of 18 specimens of *Leonia mamillare* have been cultured by the methods and the mediums employed in the study of *Cyclostoma elegans*. This series of cultures has only a comparative value. It has been pointed out that

the composition of the concretions is slightly different from that of *Cyclostoma elegans*, and it is not unlikely that on account of inadequate information, the snails, which originated from Algeria, have been kept in an environment unsuitable for their metabolism. Additional experiments are contemplated as soon as suitable specimens are available. The preparation of the snails and the culturing of the concretion deposits offers no difficulties. The shieldlike organs can be removed in toto without the least danger of contamination from the intestinal tube as follows:

Direct cultures of the concretion deposits removed from *Leonia mamillare* furnish sterile plates. Enrichment in uric acid mediums gives in 3 of 5 tests sterile transplants, while the remainder shows "fluorescens" and herbicola types, which cannot be distinguished from the bacteria isolated from the intestinal canal of the same individuals. Furthermore, these types are similar to those found on the plates prepared from the concretion deposits of *Cyclostoma elegans*. Morphologically, the gram-negative rods composing these colonies have a slight resemblance to the intracellular bacteria demonstrated in the purinocytes of *Leonia mamillare*. It must, therefore be concluded that they have not been cultivated. However, it is evident that gram-negative intestinal organisms, probably acquired with the food or water, invade the concretion deposits, and by using proper enrichment methods these bacteria can be demonstrated. This series of cultures emphasizes again the care which should be exercised in the interpretation of bacteriological findings made on the concretion deposits of *Cyclostoma elegans*. In future cultural attempts, it is advisable to use mediums that contain a mixture of various purine bases. Furthermore, the ethnology of these mollusks should be carefully reproduced in the laboratory, or specimens should be cultured as soon as possible after collection from their colonies. It is self explanatory that the intestinal flora should always be compared with that of the concretion deposits.

TAXONOMICAL, BIOCHEMICAL AND SEROLOGICAL STUDIES MADE WITH
THE PREDOMINATING TYPES OF BACTERIA ISOLATED FROM
THE CONCRETION DEPOSITS OF *CYCLOSTOMA ELEGANS*

The colonies which developed on the plates seeded with the fragments of the concretion deposits were tested in accordance with the "Manual of Methods" of pure culture study of The American Society of Bacteriologists, 1922-23; some were studied biochemically and serologically. It is not the purpose of this chapter to record the details of these tests, but to explain briefly the classification of the gram negative bacilli which has been chosen. The bacterioscopic study of the intracellular organisms of the purinocytes conveyed the impression that the infection is monobacterial. The colonies which grew on the plates, however, showed not only differences in pigmentation, size and texture, but the purified colonies produced different changes in gelatine, carbohydrate mediums, milk, etc. At first, it was thought that at least 10 to 12 different types of bacilli of identical microscopic appearance and behavior toward the gram stain were present in the concretion deposits.

In the course of the investigation it was noted that some of the purified strains when plated and examined in isolated colonies exhibited variations similar to those observed on the original plates; in fact, it became more and more evident that bacteria, which were originally considered new species, were mutants of a common genus. The 526 cultures carefully examined have been placed in 4 large groups. It seems likely that further studies may reduce the number of groups to two. Furthermore, it may be necessary to reclassify the intracellular bacteria according to their physiological activity, in which case only one group deserves consideration. The bacteria thus far found in the concretion deposits belong to a clan of micro-organisms which is widely disseminated in soil and water and on roots and plants. Some of their mutations have in the past been described as separate species, and are still maintained as such in some of the books on determinative bacteriology. I am of the opinion that the variations have been given too much classificatory value, as compared with precisely similar deviation in higher forms of life. There is no doubt that soil and water bacteria possess a remarkable plasticity and adaptability to diverse conditions of life. Intracellular existence of bacteria originally saprophytic, unquestionably stimulates the production of mutations and variations. The disappearance of the power to liquefy gelatine or to produce pigment have been observed on the cultures obtained from the concretion deposits of *Cyclostoma elegans*. Taxonomically important and conspicuous characters have disappeared and the inexperienced would be justified to classify the bacterium as a new species. Only painstaking and critical studies may reveal the parent form of the isolated micro-organism. Workers who investigate the symbiotic bacteria of invertebrates should fully appreciate these facts and should realize that the number of fluctuating types is practically infinite. This attitude may justify the classification of the bacteria isolated from the concretion deposits into broad groups rather than final identification with definite genera or species.

TAXONOMICAL STUDIES

The four groups of gram-negative bacilli isolated from the plates smeared with fragments or the enrichment cultures of concretions deposits present the following characteristics:

A "Fluorescens" Group.—Gram-negative bacilli producing a greenish, water-soluble pigment on solid and in liquid mediums, slight to moderate growth in uric acid mediums; they grow aerobically at 20 to 25 C. Mutations in the texture of the colonies are common. The

production of alkaline split products and the formation of crystals in the mediums may vary considerably. The variations are in conformity with the observations reported by Eisenberg. Two types have regularly occurred on the plates prepared from the same concretion deposit or even from a purified culture.

Type A: Fine, long, slender, sluggishly motile rods, frequently in pairs and short chains; growth on agar in thick, viscous, slimy colonies; form phosphate crystals in solid mediums; may or may not liquefy gelatine and may or may not produce acid in glucose mediums: indol is not formed. The milk shows an alkaline reaction. Ammonia is produced on blood plates. Nitrates are reduced to nitrites. This type is probably identical with *Bacterium putidum* (Flügge) Lehmann and Neumann (p. 414) or *Pseudomonas putida* (Flügge) Bergey's Manual (p. 128) or *Pseudomonas nonliquefaciens* (Eisenberg) Bergey's M. (p. 132).

Type B: Coli-like to slender, very motile rods; colonies fine, transparent with tendency to spreading. As a rule, fluorescent, greenish pigment is produced quickly and abundantly on suitable mediums. Liquefaction of gelatine and acid production in glucose; indol is not formed. Litmus milk becomes alkaline. This type is probably identical with *Bacterium fluorescens* (Flügge) Lehmann and Neumann (p. 411) or *Pseudomonas fluorescens* (Flügge) Bergey's Manual (p. 126).

B. "*Alkaligines Group*."—Small, coli-like rods in liquid mediums; coccoid bacilli resembling *Brucella melitensis* on solid mediums, motility marked to moderate or absent. Colonies on agar coli-like, sometimes slimy or thin, whitish to gray; on prolonged incubation on blood plates hemolysis. Gelatine not liquefied, no gas or acid production in carbohydrates; rapid or slow alkali-production in the milk, no coagulation. Some strains emanate a strong odor of ammonia and amines. The majority of the cultures placed in this group resemble morphologically and biochemically *Bacterium fluorescens nonliquefaciens*, which has lost its pigment-producing properties. Others are indistinguishable from *Bacterium alkaligines* (Petruschky) Lehmann and Neumann (p. 357) or *Alkaligines fecalis* (Castellani and Chambers), Bergey (p. 234). It is not unlikely that some of the strains can be classified into the genus *Achromobacter* gen. nov. Bergey (p. 132-153). Colonies of these organisms developed as a rule in association with representatives of the *fluorescens* or *herbicola* group. It seems not unlikely that some strains are mutants of these organisms. The same view has been

expressed by J. Strecker in his study of 30 strains of *B. alkaligines*. They produce ammonia in peptone solutions and milk, but they are unable to use uric acid as the only nitrogen source.

C. "Herbicola" Group.—Short, coli-like to slender rods with slight to marked motility. The colonies have on all the mediums tested a citron to golden yellow color; the gelatine is, as a rule, slowly softened or distinctly liquefied. On liquid mediums the pellicle formation is marked; glucose may show after 14 days' incubation a moderate degree of acidity; alkali production in peptone solutions containing di- and polysaccharides is distinct; the milk remains unchanged. Some of the strains grow only at 22 C., while others develop also at 37 C. Zooglea formation has been seen on 5 strains. A number of cultures characterized by a golden-yellow pigment of the colonies has been placed in this group. Prof. M. Duggeli, who saw some of the agar cultures, told me that they resembled *Bacterium herbicola aureum* (Burri and Duggeli), Lehmann (p. 394). For the sake of convenience this term has been used for all the yellow pigment-producing forms, although it is not unlikely that some of the strains placed in this group are mutants of *Bacterium putidum*, which have lost their property to produce a fluorescent pigment.

It may be a peculiar coincidence, but Beijerinck and others have frequently found *B. herbicola* associated with *B. fluorescens liquefaciens*. The same condition exists in the concretion deposits. A number of strains of the "herbicola" group, when plated on agar showed a difference in the structures of the colonies, which in many respects resembled the "flavus" or "coloides" mutants of *B. herbicola* described by Beijerinck. When working continuously with the strains of this group, one gains the impression that they are variations of A and B, except two cultures, which may possibly be identical with *Flavobacterium diffusum* (Frankland) Bergey's manual (p. 100).

D. "Colon Group."—Gram-negative, short rods with varying motility; no liquefaction of gelatine; grayish colonies; produce gas and acid in glucose, lactose, maltose and mannite; reduce neutral red, coagulate milk, form indol; grow better at 22 than at 37 C. The cultures placed in this group are typical and are probably identical with *Escherichia coli*, Bergey's "Manual" (p. 196). Rarely more than one or two colonies developed on the plates prepared from the concretion deposits. They were found more frequently in the cultures prepared from the intestinal canal. The gram-negative cocci occasionally

recovered from the concretion deposits and the gram-positive rods and cocci of the intestinal canal have not been studied in detail.

Groups A, B and C have been designated as "the alkali-producing Group." This term is merely used to classify quickly and conveniently the various cultures which accumulated in the course of the study. Representatives of this group produce alkaline split products in milk and peptone solutions; they emanate an odor of ammonia and present a hemolytic zone on blood plates. Morphologically, the organisms of these groups resemble the intracellular bacteria of the purinocytes; they form aggregations and clusters of gram-negative rods similar to those observed in smears prepared from the deposits. However, it has already been pointed out that a definite identification is not justified for the following reasons: 1. The plates seeded with large fragments of the concretion deposits develop frequently less than 10 colonies. 2. Gram-negative bacteria common to the intestinal canal are found in

TABLE 6
SUMMARY OF THE TYPES OF BACTERIA ISOLATED FROM THE CONCRETION DEPOSITS OF
CYCLOSTOMA ELEGANS

Seasonal Stages of <i>Cyclostoma elegans</i>	"Alkali-Producing Group"			Coli Group	Gram- Neg- ative Cocci	Sterile	Con- tami- nated
	"Fluor- escens"	"Alka- ligines"	"Herbi- cola"				
Hibernating..... 56	16	7 (1)	6 (2)	8 (3)	3	21	1
Active summer forms.. 68	40 (6)	3 (19)	4 (6)	8 (8)	6	1	—
Total..... 124	56 (6)	10 (20)	10 (8)	16 (11)	9	22	1

The figures in parentheses indicate that the colonies of the organisms were associated with other gram-negative bacteria; for example, "fluorescens" predominated but "alkaligenes" was also present.

the cultures prepared from the connective tissue harboring the concretions. 3. These bacteria are morphologically and bio-chemically identical. 4. In numerous instances two, even three different types of gram-negative bacteria are found in the cultures, although the microscopic examination of the purinocytes revealed only one type. In the light of these facts, it is evident that the concretion deposits of *Cyclostoma elegans* harbor micro-organisms common to their intestinal canal. However, it was of interest to know the numerical relationship of the various types. The answer to the question is given in table 6.

The data in table 6 indicate that the representatives of the "fluorescens group" are found more frequently than any other gram-negative bacteria. Hibernating as well as active summer forms of *Cyclostoma* give cultures of these types. If one accepts the views previously

advanced that most of the "alkaligines" and "herbicola" types are mutants of the *fluorescens* group, it is evident that approximately 70% of the mollusks harbor in the concretion deposits a specific group of bacilli. It became clear at this stage of the investigation that an identification of these organisms with the intracellular symbiotes would be facilitated by an analysis of the biochemical functions of the bacteria. Most of the studies on symbiosis emphasize the reciprocal advantages resulting from the associations of molds, yeast or bacteria with plants or animals. In fact, it is assumed that the symbiotes meet some physiological deficiency; either they render cellulose more readily assimilable or they supply the host with nitrogenous substances, or they furnish ferments that aid in the digestion of sugars. The intimate association of the symbiotes with the concretions composed of purine bases, primarily uric acid, appeared sufficiently significant to propose the question: "Can the cultures, particularly, the representatives of the *fluorescens*" group, decompose uric acid?"

BIOCHEMICAL STUDIES

As early as 1872, Lex reported on the fermentation caused by micro-organisms. He exposed a uric acid medium to an infection with bacteria of the air at a temperature of from 20-30 C. The purine base disappeared, and urea and ammonium carbonate were demonstrated in the solution. During the years 1886 to 1890, F. and L. Sestini conducted experiments on the ammonical fermentation of uric acid. They confirmed the work of Lex, and they showed that "*Bac. arce*" and *Bacterium fluorescens liquefaciens* convert uric acid completely into ammonium carbonate and CO_2 . If the experiment were interrupted before the uric acid had been completely destroyed, urea could be demonstrated in the solution. Burry, Herfeld and Stutzer, Gérard and Ulpiani enhanced these observations. Thus Ulpiani was able to show that one molecule of uric acid was split into 2 molecules of urea and 3 molecules CO_2 by a motile, nonsporulating "cocco-bacterium." P. Nawiaskey (1908) seeded a solution of 2 gm. of uric acid with 3 gm. of a bacterial culture of "*Bac. proteus*" and found that after 6 days 7.74 per cent. of the nitrogen of the purine base was demonstrable as ammonia. F. Liebert studied in 1909 the decomposition of uric acid by micro-organisms. He inoculated uric acid solutions with garden soil or mud and found as decomposition products allantoin, oxalic acid and urea, later CO_2 and ammonia. He repeated the experiments with pure cultures of "*Bac. fluorescens liquefaciens*," "*Bac fluorescens non-*

liquefaciens," "Bac. calco-aceticum" and "Bac. pyocyaneus," which he had isolated from the seed material used in the first series of tests. Again urea and as intermediary split products, allantoin, together with oxalic acid, was found in the cultures containing uric acid. F. Löhns, in reviewing the work on uric acid fermentation by bacteria, states that the ability to split purines, particularly uric acid, is frequently encountered in the group of the gelatine-liquefying, fluorescent, pigment-producing rods.

This brief summary indicates that bacterial species occur in nature, which can use uric acid as the sole carbon and nitrogen source. As split products urea, allantoin, oxalic acid and ammonia have been found. Preliminary observations have established the development of the "fluorescens group" in uric acid mediums, but additional experiments are necessary to demonstrate the chemical nature of the split products. Two series of biochemical tests have been carried out.

(1) *Qualitative Experiments.*—Twenty strains of "fluorescens" cultures which belong to Type A and B were inoculated into 10 c.c. of a uric acid medium (uric acid 0.5 gm.; Na_2HPo_4 , 3 gm.; salt solution, 50.0 c.c. in 450 c.c. of distilled water). After an incubation of 10 days at 22 C., 5 c.c. of the culture were tested with Fosse's xanthidrol reagent (5 c.c. medium + 10 c.c. glacial acetic acid + 3 c.c. of 10% xanthidrol in methylated alcohol). The remainder of the medium was titrated with phenol red.

The cultures were all strongly alkaline; the P_H varied between P_H 8.2-8.4; on the addition of xanthidrol, a heavy precipitate of urea-xanthidrol crystals formed in every tube, while the controls remained perfectly clear.

(2) *Quantitative Experiments.*—Five hundred cubic centimeters each of the uric acid medium used in the first experiment were inoculated with one-fifth of an agar slant of culture 98,001 and 98,002. The strains had developed in pure culture on the plates seeded with the concretion deposits of a fresh *Cyclostoma* collected at Liestal; 98,001 was a type A, gelatine nonliquefying, and 98,002 a type B, gelatine liquefying, strain.

The cultures were incubated in large 2 liter flasks at 26 C. At various intervals, Miss E. Wagner of the Hooper Foundation tested quantitatively small samples for uric acid,* amino, urea, and ammonia nitrogen.* The results are summarized in table 7.

A second series of cultures in uric acid medium with and without glucose were prepared with the same strains. The results of this experiment are summarized in table 8.

The qualitative, as well as the quantitative, tests leave no doubt that the "fluorescens" strains can support their growth in mediums con-

* The determinations have been made according to the procedures given in the Manual of Selected Biochemical Methods by F. P. Underhill, in particular the following methods have been used: Uric acid determination: Folin's uric acid method (1922). Urea determination: Marshall's urease method (1913). Amino-nitrogen determination: Van Slyke's quantitative determination of aliphatic amino groups (1913). Ammonia nitrogen: Folin's aeration method using NaOH (Am. J. Phys., 1915, 13, p. 45).

TABLE 7
"B. FLUORESCENS ON URIC ACID MEDIUM"

	Total Nitro- gen, Mg. per 100 C c.	Non- protein Nitro- gen, Mg. per 100 C c.	Uric Acid, Mg. per 100 C c.	Amino Nitro- gen, Mg. per 100 C c.	Urea Nitro- gen, Mg. per 100 C c.	Am- monia Nitro- gen, Mg. per 100 C c.	P _H	Appear- ance
Control.....	33.2	x	102.0	None	0.9	None	7.4	Crystal clear
18 hrs. { Strain 19001	x	x	78.0	x	5.8	None	7.4	Very slight turbidity
{ Strain 19002	x	x	77.2	x	6.7	None	7.4	Very slight turbidity
36 hrs. { Strain 19001	x	x	0.43	x	23.5	None	7.6	Turbid (whitish)
{ Strain 19002	x	x	0.40	x	25.3	None	7.6	Turbid (whitish)
4 days { Strain 19001	33.2	24.1	Trace	None	25.6	1.9	8.2	Green blue tinge
{ Strain 19002	33.2	30.7	Trace	None	28.3	2.1	8.2	Green blue tinge
7 days { Strain 19001	x	x	None	x	x	3.4	8.4	Green blue tinge
{ Strain 19002	x	x	None	x	19.0	4.1	8.4	Green blue tinge

TABLE 8
URIC ACID MEDIUM WITH AND WITHOUT GLUCOSE

	Total Nitro- gen, Mg. per 100 C c.	Non- protein Nitro- gen, Mg. per 100 C c.	Uric Acid, Mg. per 100 C c.	Amino Nitro- gen, Mg. per 100 C c.	Urea Nitro- gen, Mg. per 100 C c.	Am- monia Nitro- gen, Mg. per 100 C c.	P _H	Appearance
Control.....	33.2	x	102.0	None	0.09	None	7.4	Crystal clear
72 hrs. { 0.1% glucose	x	23.9	20.8	x	13.5	None	7.6	Turbid, slight greenish color
{ Strain 19002 } 0.0 glucose	31.8	30.0	None	x	23.5	None	7.6	Turbid
4 days { 0.15% glucose	x	x	Trace	x	18.8	3.4	8.2	Turbid, quite green
{ Strain 19001 }								

URIC ACID REPLACED BY ASPARAGINE

Control.....	x	x	x	12.2	x	1.4	7.4	Crystal clear
60 hr. strain 19002.....	x	x	x	2.0	0.4	12.2	8.2	Turbid, green- ish

Medium prepared in exactly the same way as uric acid medium, 0.5 gm. asparagine replacing 0.5 gm. uric acid.

taining uric acid as the only nitrogen source. In the course of their multiplication, urea appears in the medium. In the 36th hour of incubation, practically the entire uric acid nitrogen is converted into urea nitrogen. On the 7th day, the purine base has been destroyed completely, and some of the urea has been split into ammonia. In the presence of glucose, the growth is much heavier; chemical activity progresses more rapidly, with a corresponding conversion of urea into ammonia, and fixation of the nitrogen in the bacterial bodies takes place. These analyses enhance the observations of Sears, who found that *B. pyocyaneus* and *Vibrio cholera* are capable of destroying this purine base. According to recent statements, the transformation of uric acid to urea is considered a complicated and less rapid process than the conversion of urea to ammonia. This phase of the chemical activity of the "fluorescens" bacteria has not been investigated, but it is clear that at least in the experiments here recorded the process has been very rapid, particularly in the presence of glucose, and it is quite likely that allantoin is not formed as an intermediary product. Little need be said regarding the hydrolysis of the urea to ammonia. To Schellmann must be attributed the statement that uric acid fermenters are equally active urea splitters, and vice versa. Stapp has shown that some of the gram-positive, spore-bearing, uric acid-splitting bacteria grew better in the presence of dextrose. The same worker has noted development of his strains on mediums containing xanthine, hypoxanthine, guanine, allantoin, alloxane, etc., as the only nitrogen source. Future biochemical studies with the uric acid-splitting bacteria of the concretion deposits should consider these observations.

Ten strains of the "alkaligines," 5 of the "herbicola" and 2 of the "colon" group were tested on the uric acid medium. Eight of the alkaligines and all of the "herbicola" colon strains failed to grow. For reasons to be discussed later, it appeared important to know whether these inactive strains were capable of development in mediums containing the split products of the "fluorescens" group. Ten-day old cultures of several "fluorescens" strains were filtered through Berkefeld candles, tested for sterility and inoculated. In comparison with cultures containing peptones, a scanty development was noticed. The medium was free from uric acid, but contained a small amount of urea and ammonium salts. Additional tests, which need further confirmation, have shown that the "alkaligines" and colon strains grow best on mediums containing albuminous material and amino-acids. An extract of *Cyclostoma* snails gave excellent cultures and an abundant production of

alkaline-split products. These cultures when filtered furnished a perfect substratum for the "fluorescens" strains. In this connection, it is recalled that Liot obtained pigment production and Braun and Cahn-Bronner noted excellent growth of *B. pyocyaneus* on mediums containing ammonium salts. This observation has been confirmed for the fluorescens strains isolated from *Cyclostoma elegans*. For example, a phosphate solution with ammonium carbonate (0.5 per cent.) and levulose (0.1 per cent.) gave good growth and pigment production. It was, however, noted that cultures in mediums composed of uric acid and ammonium nitrate were less vigorous than those in pure uric acid-phosphate solutions. Stapp has made similar observations with gram-positive uric acid bacteria; in the presence of ammonium salts, bacterial uricolysis is apparently inhibited.

Qualitative tests conducted with several "alkaligines strains" in urea-phosphate solution confirmed previous observations made in series 9. These organisms grow readily in such mediums; they hydrolyze the urea to ammonia.

These test-tube experiments throw some light on the mutual relationship of the gram-negative bacteria isolated from the concretion deposits. The "alkaligines strains" produce in the course of their growth ammonium salts, which are used by the "fluorescens types." These in turn are capable of attacking the purine bases converting them into urea, ammonia and CO_2 . The biochemical behavior of the pure cultures of gram-negative bacteria secured from the concretions of *Cyclostoma* lends some support to the conclusion that the cultivated micro-organisms are identical with the intracellular symbiotes of the purinocytes. In this connection it should, however, be emphasized that strains of the "fluorescens" and "alkaligines" group isolated from the intestinal tract of the mollusks acted in the same manner as the strains cultured from the concretions. A distinction between the intestinal bacteria and the symbiotes has not been accomplished by biochemical tests. Serological procedures have therefore been chosen to solve the question of identification.

STUDIES ON FERMENTS

The biochemical studies have conclusively demonstrated the ability of the "fluorescens" types to destroy uric acid. It appears of interest to determine the nature of the enzymes responsible for the conversion of the purine base to urea. Little is known regarding the occurrence of purinolytic bacterial ferments. Waksman in his excellent review mentions Straughn and Jones who found that certain yeasts formed guanase,

which acts upon guanine, with the formation of xanthine and ammonia. Kossowicz has shown that molds, including *Aspergillum niger*, produce an enzyme which acts on uric acid and hippuric acid, with the formation of ammonia. No reports dealing with the formation of urease by the *fluorescens* bacteria are available, although the recent work by Lövgren indicate that all micro-organisms are able to decompose urea to some extent. The following experiments, which must be considered preliminary tests, suggest the formation of an uricolytic ferment by strain 98001, when grown on a medium containing uric acid as the sole nitrogen source.

Experiment 1.—Strain 98,001 was grown on uric acid agar and in a liquid medium of identical composition. The moderately heavy growth was washed off or concentrated by centrifugalization, suspended in salt solution, pooled and washed once with salt solution. The sediment was added directly to the uric acid solution, or it was treated with absolute alcohol and ether and rapidly dried over H_2SO_4 . The dried material was ground in an agate mortar and the used for the experiments.

One cubic centimeter of the suspension or 10 mg. of the dried material were added to 25 c.c. each of an approximately 0.5 per cent. uric acid solution in distilled water. This mixture was kept with 3 per cent. toluene, in shallow layers, at a temperature of 22 C. for 10 days. As controls were used distilled water with a suspension or dried bacterial material and an uninoculated flask of test solution. Filtered specimens were tested for urea by the xanthidrol method, for ammonia by determining the P_H and with Nessler's reagent.

The flask inoculated with the dried bacteria gave a distinctly alkaline reaction (P_H 8.2); the ammonia test was positive and the xanthidrol reagent produced a heavy precipitate of typical dioxanthidrol-urea crystals.

Experiment 2.—Twenty-five cubic centimeters each of a solution of approximately 0.04 per cent. uric acid were inoculated with 5 c.c. of a heavy growth of *fluorescens* 98,002 from an ordinary peptone-agar slant or 5 c.c. of a 5-days broth culture of the same organism. The mixtures were carbolyzed (0.5 per cent.) and incubated at 22 C. for 5 days.

The uric acid determinations gave the following readings:

Test solution, 35.4 mg. per 100 c.c.; urea tests negative.

Agar growth, 37.5 mg. per 100 c.c.; urea tests positive (from culture).

Broth culture, 37.5 mg. per 100 c.c.; urea tests positive (from culture).

Result:—No uric acid splitting enzyme was demonstrated.

These two experiments should be enhanced by further tests. It is evident that dried and ground bacteria contain an intra-cellular enzyme which converts uric acid into urea and ammonia. This action has not occurred when bacteria killed by carbolic acid or inhibited in their growth by toluene have been used. The uricolytic enzyme apparently exerts his influence during cell growth and is probably intimately connected with vital bacterial activity.

Serological Tests.—Serological methods have rarely been employed in the study and identification of symbiotes.* Brues and Glaser have recently reported on the use of the precipitin and agglutination test to identify a fungus of the *Dematium* type isolated from the fat body of the cotton maple scale, *Pulvinaria innumerabilis*. They inoculated rabbits with pure cultures of the fungus and tested the serum of these animals against the yeastlike cells isolated from the living scales. The reaction was distinctly positive, but not so pronounced as with the fungus in culture. A somewhat different procedure has been chosen for the concretion deposits of *Cyclostoma*. The experiments are herewith reported in detail.

Series 1-2: Rabbits were inoculated intravenously with the bacterial suspensions prepared from the purinocytes. The concretion deposits of 37 *Cyclostomas* used in culture series 10 and 11 were suspended in salt solution, crushed with a glass rod and shaken for 2 hours. The suspension was centrifugalized for a few minutes, the supernatant fluid removed, strained through cotton and centrifugalized until clear. The sediment, consisting mainly of symbiotes, was resuspended in fresh salt solution, and the process repeated twice. The washed bacteria were suspended in formalinized salt solution (0.1 per cent. formaldehyde) and used for the inoculation of the animals. Three injections of 2 c.c. suspension at 3-day intervals were given. The rabbits, which gained in weight, were bled from the heart on the 10th day after the last injection. The serum of one rabbit agglutinated the bacteria of the suspension used for the inoculations in a dilution of 1:40 +++ in two hours at 37 C. and 1:80 +++ in 24 hours at room temperature. Normal rabbit serum failed to agglutinate the bacteria in a dilution of 1:5.

The specific agglutinating serum was diluted 1:20, and 2 c.c. amounts were pipetted into small tubes. A similar series was prepared with normal rabbit serum diluted 1:5. Into one tube each of the two series was carefully rubbed the 48-hour old growth on agar slants of 67 pure strains of gram-negative bacteria isolated from the concretion deposits of the series 10 and 11. Forty strains of the "fluorescens," 18 of the "alkaligines," 5 of the "herbicola" and 4 of the "colon" group were used in this test. No specific agglutination reactions were obtained. Three strains were agglutinated by the immune and by the normal serum. Equally negative tests were secured with 105 cultures isolated from cultural series 1, 2, 3, 4, and 8.

The agglutination test gave therefore a negative result and failed to identify the cultures with the intracellular bacteria, although the serum employed contained specific antibodies for the symbiotes. Subsequent serologic tests detailed in series 2 and 3 may explain these results.

* J. W. Stevens (Jour. Infect. Dis., 1923, 33, p. 557) has recently applied serologic tests to the study of legume bacteria.

Series 2: In this series, the serological relationship of the representatives of the "fluorescens" group was investigated. Two strains of "fluorescens" 98,001 (gelatine liquefied, type A) and 98,002 (gelatine nonliquefied, type B) isolated from the same plate and the same snail were used. Rabbits were immunized with dead and living cultures. One intraperitoneal and 2 intravenous injections were given. The serums agglutinated their homologous strains in a dilution of 1:100—200 at 37 C. for two hours and 1:200—1:400 at room temperature for 24 hours. In a dilution of 1:50 the bacteria agglutinated promptly when examined microscopically. Cross agglutination did not take place, even in a dilution of 1:5; in other words, the serum specific for strain 98,001 never clumped the living or formalinized suspension of strain 98,002, and vice versa. The serums possessed a high degree of specificity on account of the few inoculations which were given to the rabbits. The results are quite in harmony with the findings of Pribram and Pulay, who found that the *Bac. fluorescens liquefaciens* and *Bact. putidum* are not related. Furthermore, through the publications of Klieneberger, Jacobsthal and others, it is known that the different strains of *B. pyocyaneus* cannot immunologically be classed into a uniform group. One is then confronted with the fact that in the concretion deposits two strains of bacteria possess the same pigment and the same ability to split uric acid, but they are not related serologically. This example serves to emphasize from another angle the complexity of the problem of identification. It was important to ascertain whether the intestinal strains were immunologically identical or different from the concretion strains of the same snail. Twelve cultures of two snails, 93 and 94, were tested, with entirely negative results. Specific serums were prepared with one intestinal strain of mollusk 93 and one concretion strain of *Cyclostoma* 94. The findings are summarized in table 9.

TABLE 9
CROSS AGGLUTINATION OF FLUORESCENS STRAINS

Serum prepared with intestinal strain 93 (3) (<i>B. fluorescens liquefaciens</i>), tested against	Heterologous culture from concretion deposit 1; agglutination	0
	Heterologous culture from concretion deposit 2; agglutination	0
	Heterologous culture from concretion deposit 3; agglutination	0
	Heterologous culture from intestinal content 1; agglutination	0
	Heterologous culture from intestinal content 2; agglutination	0
Serum prepared with concretion strain 94 (1) (<i>B. fluorescens liquefaciens</i>), tested against	Homologous culture from intestinal content 3; agglutination	1:100
	Homologous culture from concretion deposit (1); agglutination	1:400
	Heterologous culture from concretion deposit (2); agglutination	0
	Heterologous culture from concretion deposit (3); agglutination	0
	Heterologous culture from intestinal content (1); agglutination	0
	Heterologous culture from intestinal content (2); agglutination	0
	Heterologous culture from intestinal content (3); agglutination	0

This high degree of specificity was not expected in the light of the recent studies reported by Pribram and Pulay, at least a serological relationship between typical strains of *B. fluorescens liquefaciens* was anticipated. The actual tests, nevertheless, indicate the existence of immunologically nonrelated species, even in a biochemically well defined and uniform group. There was still a possibility that some of the "fluorescens" strains isolated from the concretion deposits and the intestinal tract of *Cyclostoma elegans* living in the same locality were identical. One is here reminded of the view advanced by Mercier

(p. 38) that the bacteria of the purinocytes may enter the cells from the intestinal canal, which has become invaded through the food previously contaminated with fecal excreta or decomposed mollusks heavily parasitized with "specific" intracellular bacteria. Ninety-six strains of the "fluorescens group" isolated from the concretion deposits of mollusks collected at Liestal, Geneva, Grenoble, Marseilles and Mendrisio have been tested with 4 different immune and one normal rabbit serum. Again, serum dilutions (1:50) have been used. The serum prepared with strain 98001 agglutinated specifically one culture (1082) secured from a mollusk collected at Grenoble, while that of strain 98002 clumped two cultures (963 and 93002) obtained from concretion deposits of *Cyclostoma* found at Liestal. The remaining 93 cultures representatives of types A and B were not affected by the serums. The three strains (1082, 963 and 93002) were likewise not agglutinated by the other two serums prepared with the intestinal strain 93 (3) and the concretion strain 94 (1).

From these results, it is logical to hold that the "fluorescens group" is composed of numerous immunological sub-groups which cannot be recognized by a monovalent serum. It is difficult to surmise how many of these groups actually exist; the tests are sufficiently conclusive to show that numerous representatives of these groups may be isolated from the concretion deposits or the intestinal tract of *Cyclostoma*. A specific "fluorescens" type common to the snails of a certain locality has not been found. This fact is analogous to a condition frequently met with in bacteria present in soil and water. It may serve as an additional proof for the observations previously recorded; plasticity and variability is a characteristic feature of the bacteria found in the concretion deposits. One is generally inclined to assume by analogy from work done with pathogenic micro-organisms that intimate, intracellular habitat stabilizes the bacterial type and creates immunologically fixed forms. This deduction is probably too broad, and the conception of fixed types cannot be applied to the existence of bacteria in the cells of poikilothermic invertebrates. In fact, it is quite likely that the cultivated micro-organisms may be accidental invaders of the concretion deposits. They belong to many different groups and behave like any other soil or water bacterium. As an example of the latter condition, the recent studies on grouping of *B. botulinus* could be mentioned (Starin and Dack, Schoenholz and Meyer).

The serological studies on the "fluorescens" group were so decisive that it appears superfluous to record a few tests that have been made

with representatives of the "alkaligines" group. They are merely recorded for the sake of completion.

Series 3: Two strains (964 and 967) belonging to the "alkaligines group" and isolated from the concretion deposit of a *Cyclostoma* found at Liestal (May, 1923) were chosen for the immunization of two rabbits. After three injections, the serum agglutinated the homologous strains in a dilution of 1:100 and 1:200, respectively. No cross agglutination was obtained: 964 did not agglutinate 967, and vice versa. Eleven strains of the "alkaligines group" were tested macroscopically in serum dilution of 1:50 and 1:100. Negative results were recorded throughout.

These tests, relatively few in number, conclusively demonstrate that the "alkaligines" group is serologically composed of a large number of subgroups. The discussion presented in connection with the findings in the "fluorescens group" can unreservedly be transferred to the results just reported. In fact, it can be applied to the entire series of serologic tests which were instituted to establish the identity of the cultivated bacilli with the intracellular symbiotes. The question, "Why did the antisera which agglutinated specifically the intracellular bacteria fail to give reactions with the pure cultures?" can now be discussed: (a) Either the intracellular organisms have not been cultivated, and therefore no reactions can be expected with the cultures. (b) The symbiotes are composed of a heterogeneous agglomeration of biochemically identical gram-negative rods, each group only represented in the concretion deposits by antigenically low, but highly specific, types, which stimulate few or no agglutinins. Experimentally, the best conditions have been chosen. Cultures which were secured from concretion deposits were used for the immunization of the rabbits. It would be a simple matter to dispose of the uncertainty by accepting the first of the two possibilities. However, the frequent cultivation of a group of bacteria morphologically identical with the symbiotes and, probably, the most active of the class of purines-splitting micro-organisms, gives equal right to the second explanation. It should always be kept in mind that the study of the intracellular bacteria of *Cyclostoma* represents the first systematic effort to classify by bacteriological methods and to analyze by analogy deductions a phenomenon peculiar to invertebrate animals. Until further investigations have broadened the field and the physiological significance of intracellular symbiotic habitat has been established by carefully controlled experiments, it appears advisable to formulate a working hypothesis and to accept the cultures as those of the symbiotes, irrespective of the negative or inconclusive serological findings.

SUMMARY AND DISCUSSION OF THE BACTERIOLOGICAL FINDINGS MADE
ON THE CONCRETION DEPOSITS OF *CYCLOSTOMA ELEGANS*,
CYCLOSTOMA SULCATUM AND *LEONIA MAMILLARE*

The studies to cultivate the bacteria demonstrated in the concretion deposits have confirmed the observations of Garnault and Mercier; gram-negative bacilli have been grown on a variety of culture mediums. However, not one particular type or one specific species has been isolated and propagated in pure culture, but representatives of at least 4 large groups of bacteria have been recognized. Furthermore, the cultures exhibit a morphological and biochemical behavior typical for certain saprophytes of the soil and water. In fact, from the standpoint of taxonomy the micro-organisms are indistinguishable from *B. fluorescens*, *B. herbicola*, *B. coli*, etc. Comparative cultures prepared with the concretion deposits and the intestinal content of the mollusks have added additional confusion to the complex problem. The flora of the gram-negative rods found in the intestinal canal contains the same types and species as some of the concretion deposits. The statement that the latter organ has a selective affinity for the gram-negative bacilli is fully justified in the light of the cultural results. Occasionally gram-negative and gram-positive cocci and gram-positive rods have been observed on the plates prepared from the purinocytes; their presence left no doubt that the intestinal flora may occasionally invade the concretion deposits.

It should be emphasized that practically identical findings have been made on mediums, whether they contained vertebrate or mollusk protein. An extract of *Cyclostoma* stiffened by agar and reinforced by the addition of *Helix* or *Cyclostoma* lymph grew the same gram-negative bacilli as a "vitamin" beef heart-rabbit blood agar.

Mercier conveys the impression that the gram-negative bacilli develop particularly well on coagulated blood of *Helix pomatia*. This claim has been investigated. The same bacteria as found on other mediums have been isolated.

The striking individual and seasonal differences in the number of colonies which grew on the plates heavily seeded with concretions have added further difficulties to the interpretation of the cultural findings. It was expected that the smearing of crushed concretions teeming with bacteria on suitable mediums would produce a confluent growth. Instead, however, sterile plates or a few scattered colonies have been obtained. About 40 per cent. of the hibernating *Cyclostoma* furnish sterile plates,

and the remainder give cultures with few colonies. Active mollusks with moist type 1 or dry type 3 concretions give similar cultures. Invariably the bacteria of the purinocytes have been found in the smears prepared from the sterile agar surfaces. These results indicate that certain individual mollusks may harbor in their tissues microscopically demonstrable, noncultivable bacteria.

In searching for an explanation, it has been noted that the bacteria found in the purinocytes stain poorly with the ordinary aniline dyes; they appear to be either dead or injured. No satisfactory method has been found which would permit of a definite distinction between dead and living bacteria, although the silver method diagnosed the intracellular bacteria as nonviable. In the presence of purine bases, this staining reaction is, however, uncertain and inconclusive. In this connection, it is remembered that a number of workers (Gotschlich, p. 83) found that dead bacteria retain their property to stain with aniline dyes. Unna has devised a special method to distinguish dead and living intracellular leprosy bacilli. It has been tried on the purinocytes without success; all the bacteria of the concretion deposits stained yellow. Every bacteriologist recalls in his experience observations in which few colonies developed, although the stained smear indicates an abundance of viable bacteria. For example, a stool specimen may show a large percentage of *B. bifidus*, which today can be quantitatively cultivated, while the next day an equally positive microscopic picture gives rise to a scanty growth. Human, animal and plant pathology record instances in which the dormant bacilli or cocci are seen in large numbers, yet when transferred to suitable mediums a few colonies develop. E. F. Smith found, for example, the organisms of grown galls to be dormant, and recommended for the isolation of *B. tumefaciens* the use of large specimens of the tumor material. It should also be kept in mind that the hibernating mollusks which give sterile plates or rare colonies have been removed from a soil with a low temperature and have been kept in dry earth in a cool room. The viability of the symbiotes is unquestionably reduced under these conditions. These arguments are by no means conclusive, and additional evidence must be presented against the view that the cultivated gram-negative rods are not intestinal contamination. One is inclined to suspect a new, characteristic bacterium as the intracellular parasite. Failing to find such an organism, the critical worker hesitates to associate a single or a group of relatively common micro-organisms with the process in which he is interested.

However, the reverse has been practised; bacteria, which are certainly contaminations, have been considered symbiotes. The hypothesis of Portier seems to be an excellent example to illustrate the latter course of reasoning. The literature dealing with the cultivation of the symbiotes in insects or the isolation of luminous bacteria from sea animals furnishes a number of interesting reports which deserve consideration in this connection.

For example, Mercier (209) reported in 1906 that he had cultivated the so called "Blöchmann bodies" of *Blattella* and named the organism *B. cuenoti*; Javelly, in 1914, obtained negative cultures while studying *Periplaneta orientalis*. American cockroaches, *Parcoblatta virginica* and *Periplaneta americana* were studied by Glaser in 1920. At first he considered the cultures of two species of pleomorphic spirilea as the symbiotes; from more recent work he is, however, convinced that he has not grown the symbiotes, but that the two organisms are simply very common nonpathogenic parasites, with which his material has become infected. Hertig, who independently attempted the cultivation of the bacteroids of the Blattidae, found that the true symbiotes resisted all attempts at propagation on artificial mediums. His evidence implies that the spore-bearing organism described by Mercier as *B. cuenoti* belongs in the group of the common contamination of cultures. This verdict may be final, but it certainly is peculiar that Mercier obtained the same organism from 40 different cockroaches.

Several workers (Buchner, Šulc, Teodoro) who have recently examined the symbiotes of coccids regard them as yeasts, although Berlese referred one species to the genus *Oöspora*, one of the hyphomycetes, and Brues and Glaser isolated from *Pulvinaria innumerabilis* a fungus belonging to the species of *Dematium* or a related genus. Lindner, who found a yeastlike organism in a European scale insect (*Aspidiotus nerii* according to Lindinger [Buchner, p. 225] a species of *Lecanium*), was unable to cultivate it, although he evidently regarded it as a parasite, naming it *Saccharomyces apiculatus*, var. *parasitus*. The cultural studies of Conte and Faucheron on the fat bodies of several species of *Lecania* (*Lec. hemisphaericum oelae*, *hesperidum*) resulted in the isolation of yeasts which resembled the symbiotes, previously found in the dissected insects. The cultural studies are, with the exception of those published by Brues and Glaser, so incomplete that it is impossible to render a final verdict as to the exact nature of the symbiotes. The inability of Lindner, an experienced mycologist to

cultivate the yeastlike bodies deserves recognition when analyzing the reports on positive cultures secured from the fat bodies of coccinae.

Three investigators succeeded in propagating the symbiotes of Aphidae on artificial mediums. Pierantoni designates them as *Saccharomycetes*, Sulç as *Schizosaccharomycetes*, and Peklo as *Azotobacter*. The descriptions of the Peklo mention the growth of "giant cocci" changing into sarcina-like clusters or into long threads, which later divide into typical rods (?). This polymorphism is well known in the genus *Azobacter* and has been carefully discussed by Löhnis and Smith, but one gains the impression that Peklo has handled mixed cultures. In fact, the studies of this biologist on *Aphis platanoides* throw a peculiar light on the character of the bacteriological work he has published. He secured a number of bacterial strains on artificial mediums, and although he could not readily identify them with the intracellular micro-organisms, he assumes that they must be the symbiotes, because they originated from the interior of the insects. The recent studies of Pierantoni and Harvey on the luminescence in animals due to certain symbiotic organisms may be chosen as another example to illustrate the difficulties which may be encountered in correlating the microscopic with the cultural findings. Pierantoni investigated, in 1914, the luminous organs of glow-worms (*Lampyrus*) and found them to consist of parenchymatous cells crowded with minute bodies having bacteria-like staining reactions, these bodies being also present in the beetle's egg, which is luminous. He cultivated two species of micro-organisms, one resembling a rod and the other a coccus; however, the artificial cultures failed to luminate. The causal relationship is therefore not established. In 1918, Pierantoni made the discovery that luminescence in certain cephalopods, *Loligo* and *Sepiola*, is due to light-producing bacterial symbiotes living in special organ of the host. These symbiotes have been cultivated by Pierantoni and Zirpolo, but Miss S. Mortara pointed out that phosphorescent bacteria can be readily cultivated from the surface and tissues of maritime animals, although they may not be isolated from the luminous organs of the cephalopod *Heteroteuthis dispar* or the photogenic organ of *Lampyrus noctiluca*. The criticisms made by Molisch, the great authority on photogenous bacteria, indicate that care has to be exercised in proving the identity of the cultures with the microbes seen in the luminous organs. Of great interest in this connection are the researches of Harvey (1921) on the light production of two species of fish (*Photoblepharon* and *Anomalops*). The continuity of the light, independently of stimulation, is characteristic of

luminous bacteria and fungi; this, and the circumstance that luciferin and luciferase could not be demonstrated, all go to confirm the correctness of Harvey's conclusions, namely, that the luminosity of these fish appears to be due to luminous bacteria living in special organs. Cultures of the microbes have been secured by Harvey, but no light has been observed in any case. It is not unlikely that luminosity of these cultures may occur under special conditions as yet unknown. One is reminded of the work of Giard and Billet (1889-90), who succeeded in inoculating many different kinds of amphipod crustacea (*Talitrus*, *Orchestia*, *Ligia*) with luminous bacteria. The microbes do not luminesce in cultures, but do so when grown in the body of the crustacea, which succumb to the infection in about seven days. These examples indicate a tendency to choose functional and biochemical characteristics, and not morphological, taxonomical and cultural characteristics, for an identification of the symbiotes.

The cultural findings made on the concretion deposits of *Cyclostoma elegans* appear in an entirely different light as soon as the functional aspect is carefully considered. Active mollusks studied during the summer months furnish abundant cultures. Mollusks kept in captivity at a fairly high temperature (18-20 C.) in a hot-house gave similar results. Furthermore, a few active specimens of cyclostoma have been encountered in which the intestinal contents contained a few viable bacteria, while the concretion deposits give a profuse growth of gram-negative, ammonia-producing rods. Simultaneous cultures made from the lymph, the sex organs and other tissues, remain sterile, although the crushed concretion deposits may furnish innumerable colonies. The relative proportion of the three types of gram-negative rods found on the plates varies with the individual mollusks. The biochemical tests reported in detail explain the mutual relationship and the functional importance of these types of bacteria in the purinocytes. One group converts uric acid into urea and ammonia, while the other groups may utilize urea and ammonium salts in their metabolism. Concretions of the type 1 contain relatively few "fluorescens," but numerous "alkaligines" or "herbicola"-like, non-uric-acid-splitting types. This phenomenon suggests a carefully balanced relationship between the various types of the bacteria. The uricolytic bacteria ("fluorescens" types) are found in large numbers, when absorption of the concretions is initiated or in progress. There may be a delicately adjusted equilibrium between the split products of one group of bacteria with the growth of the others or with the excretory function of the purinocytes. Mutual

independence of microbic nutrition and potential microbic action occurs according to Kendall in a multitude of biological phenomena. Until one knows more about the physiology of the concretion deposits, it would be unwise to speculate regarding the factors which promote the development of one and the suppression of the other intracellular microorganisms. It is not unlikely that in the concretion deposits the alimentary environment suitable for the bacteria may undergo cyclic changes, and consequently cultures may furnish a diversity of strains, suggesting a mixed invasion of intestinal bacteria. The data thus far available leave no doubt that the gram-negative bacilli, in particular one group, can actively split uric acid and as such supply a ferment which is apparently absent from the tissues of the mollusks. This explanation is worthy of further investigation and experimentation, if one recalls in this connection the microscopic findings, which suggest that the purine bases disappear from the concretions before the latter are attacked by the amoebocytes.

In the preceding paragraph direct and indirect proof has been offered to accept the "fluorescens" types as the important cultivatable symbiotes. However, no effort has been made to trace the origin of these bacteria. The representatives of the "fluorescens" bacteria are widely distributed in nature, for example, Duggeli, Kürsteiner and others found it constantly on dead leaves. Others isolated them from water. Bornand even claims to have established their identity with *Bac. salmonicida*, the causative organism of a fatal salmon disease. Such a relationship is, however, denied by Plehn and Trommsdorff. As casual saprophytes, they have been encountered by White in the alimentary canal of bees. Where vegetables undergo decomposition, the "fluorescens" bacteria are present. If the observations of Simroth are correct that *Cyclostoma elegans* feeds primarily on decaying leaves, then the finding of the bacteria in the intestinal canal of the snails is not surprising. Their presence in the concretion deposits is, however, not clear. The striking predominance, occasionally the isolation of a pure culture from the deposits and the absence of these bacteria from other tissues of the mollusks supports the conception that the "fluorescens types" are specific invaders, irrespective of their ubiquitous and common occurrence in nature.

Aside from individual and seasonal, also geographical, differences of certain bacterial types have been noted. Certain mollusks obtained from Geneva gave practically pure cultures of colon types, while those sent from Nice and Grenoble were heavily contaminated with gram-

negative cocci. Similar findings have been made on *Cyclostoma* which have been handled in the laboratory. These invertebrates were mostly inactive; pulsation of the heart was absent or barely visible. Cocci indistinguishable from those found in the intestinal content have been isolated from the concretion deposits. As an entire series of mollusks kept in one particular terrarium furnished cultures with a predominance of cocci, it appears quite plausible that the animals suffered from an infection. This observation recalls the bacteriological findings on cockroaches reported by Glaser. In this connection mention should be made of the interesting findings recently reported by Kraus and others on *Coccobacillus acridiorum* of locusts. In 1910, F. d'Herelle observed an epizootic disease among the migratory locust, *Schistocera americana* Drury. He isolated as the casual organism *Coccobacillus acridiorum*, and he reproduced disease and death by inoculating healthy locusts with the culture. The results of his experiments led him to believe that the use of this organism would have successful results in the control of the locusts. Sergeant and Lheritier in Algeria, Lounsbury in South Africa, Barber and Jones in the Philippines, Du Porte and Vanderleck in Canada and R. Kraus in Argentine attempted to introduce the disease in order to control a pending invasion of locusts. The experiments were either complete failures or inconclusive. However, it was found by Kraus that *Coccobacillus* d'Herelle is a common intestinal inhabitant of healthy locusts. The insects can, however, be killed by intraperitoneal injections of cultures prepared with this organism. Du Porte and Vanderleck made similar observations in Canada. They isolated a number of strains of *Coccobacillus acridiorum* from healthy, diseased or dead *Melanoplus bivittatus*. Indigenous coccobacilli have also been found by Beguet, Musso and Sergeant in the locusts of Algiers. Furthermore, Glaser has found that different organisms are distributed under the name of *Coccobacillus acridiorum*. These results demonstrate that similar criteria as those used on vertebrates should be applied to the bacteriological and, particularly, the cultural findings made on invertebrates. Only specimens in perfect health and kept under sanitary conditions should be examined. This prerequisite applies primarily to those studies that deal with the isolation and identification of symbiotic micro-organisms.

Little need to be said regarding the bacteriological findings on a series of *Cyclostoma sulcatum* and *Leonia mamillare*. The cultures were merely prepared as controls for the work on *Cyclostoma elegans*. No particular efforts were made to keep the mollusks in a condition

suitable for their metabolism, nor were special mediums employed for the cultures. The results warrant the conclusions that the intracellular symbiotes have not been cultivated. An effort is being made to secure additional specimens in order to continue the bacteriological studies on a more elaborate basis.

The final decision concerning the true relationship of the cultivated bacteria to the intracellular symbiotes of *Cyclostoma elegans* must be left open. The various facts which refute and those which support their identity are herewith summarized. The observations which indicate that the intracellular bacteria have not been propagated on artificial mediums are as follows:

1. Sterile cultures are frequently secured, although the symbiotes of the purinocytes have been transferred to the mediums in large amounts, and the organisms have been demonstrated in smears prepared from the sterile plates. Individual and seasonal differences in the number of the cultivatable, but not in the microscopically demonstrable, bacteria are present.

2. The bacterioscopic examination of the purinocytes indicates a monobacterial infection, while the cultures suggest a polybacterial, at least a trisymbiotic, invasion.

3. Intestinal bacteria are frequently encountered in the concretion deposits.

4. The cultivated bacteria belong serologically to a heterologous group of micro-organisms. An immune serum produced with the intracellular bacteria failed to agglutinate the cultures.

5. The bacterial types propagated on artificial mediums are closely allied, if not identical with certain saprophytes present in the environment, the food and the intestinal tract of the mollusks.

6. The symbiotes of *Cyclostoma sulcatum* and *Leonia mamillare* have not been cultivated.

7. The symbiotes from a small number of host species (insects) have been cultivated on artificial mediums, but in most cases the artificial cultivation experiments have so far met with unsurmountable difficulties.

The following facts suggest that the bacteria grown in the cultures are the real symbiotes:

1. The microbes propagated in cultures are gram-negative; in size and arrangement they can frequently not be distinguished from the intracellular rods smeared and stained on the same slide.

2. Pure cultures, or at least a numerical preponderance of a group of bacteria, which convert uric acid into urea and ammonia have been secured. They apparently furnish uricolytic ferments, which has thus far not been demonstrated in the tissues of the mollusks.

3. The cultural studies of symbiotic processes in invertebrate animals (insects) thus far reported have usually revealed a di- or tri-microbial infection by bacteria saprophytic to the host.

A CONSIDERATION OF THE PHYSIOLOGICAL FUNCTION OF THE CONCRETION DEPOSITS

WITH NOTES ON THE RESPIRATORY QUOTIENT AND THE PRESENCE
OF UREA IN *CYCLOSTOMA ELEGANS*

The histological and bacteriological studies on *Cyclostoma elegans* appear incomplete without a brief consideration of the physiological significance and the function of the concretion deposits. The original plan of the investigation did not contemplate experiments dealing with the functional aspect of the excretory processes in the Cyclostomatidae, on account of the fact that very little is known regarding the ethology of the mollusks. No treatise similar to that published by Meisenheimer on *Helix pomatia* is available.

Cyclostoma elegans kept in captivity in a terrarium or observed in nature, where they live at the roots of trees, under stones or decaying vegetation, exhibit periods of great activity accompanied by feeding, followed by those of complete rest. Rossmässler considers the mollusk as very timid; he writes as follows:

“Hinsichtlich der Lebhaftigkeit habe ich in meinen Behältern das Gegenteil beobachtet; es sind nämlich alle meine *Cyclostomen* den Tagüber munter und lebendig; ruhen dagegen in ihrem wohlverschlossenen Gehäuse des Abends. Die Exkremente bestehen aus kleinen schwarzen Pillen.”

Aside from the daily periods of inactivity, suspended locomotion, or ingestion of food for several weeks or months (hibernation) are regular occurrences in the life of *Cyclostoma*. The humidity of the air, which is very important, notably in many terrestrial mollusks, is probably one of the factors responsible for the suspended activity. Many other causes may contribute to this peculiar behavior, and extended observations of the snail under various conditions are certainly needed before any detailed physiological studies can be undertaken. This interesting behavior of the operculate mollusks is, probably, responsible for the

remark made by Simroth that the metabolism of the snail is "slow and sluggish." No physiological evidence was, however, presented by this worker to support the statement.

While experimenting with the micro-respiration-apparatus of Krogh, modified by Jordan and Schwarz, a few comparative tests on the respiratory exchange of *Cyclostoma* and *Bulimus detritus* were suggested. Dr. A. Müller, late assistant of Prof. J. Strohl, kindly cooperated and executed the technical part of the experiments. The microrespirometer is excellently suited to determine the relative consumption of O_2 by two animals of the same volume and kept under identical environmental conditions. When combined with an estimation of the CO_2 elimination, the total catabolism can be measured. The experiments were conducted with active *Cyclostoma elegans*, recently collected at Liestal and the specimens of *Bulimus detritus* secured through the kind assistance of Prof. J. Strohl at Glattfelden, as shown in table 10.

TABLE 10

RESPIRATORY EXCHANGE AND QUOTIENT OF *BULIMUS* AND *CYCLOSTOMA* DETERMINED BY THE MICRORESPIROMETER OF JORDAN AND SCHWARZ

Bulimus detritus							Cyclostoma elegans						
Ex- peri- ment	Speci- men	Weight	Duration of Experiment		CO ₂ Elimi- nated, %	O ₂ Con- sumed, %	Temperature, 15-18 C.	Speci- men	Weight	Duration of Experiment		CO ₂ Elimi- nated, %	O ₂ Con- sumed, %
			Hr.	Min.						Hr.	Min.		
1	1	0.83	3	..	1.6	3.7		1	0.75	5	..	1.0	9.4
2	1	0.86	8	..	1.6	2.2		1	0.82	8	..	(-0.4)	2.5
3	1	0.86	8	0	2.1	4.7		2	0.98	4	15	2.6	3.2
4	1	0.9	6	40	4.1	3.3		1	9.62	6	40	0.3	0.6
5	1	0.78	6	45	0.4	4.3		1	0.62	6	40	0.4	2.5
6	1	0.92	15	10	8.2	15.3		1	0.88	14	45	1.3	4.8
7	1	0.94	15	..	12.0	16.5		2	0.92	15	..	2.5	6.3
8		2	0.92	15	25	3.6	6.9
Average of 1 hour per animal: $\frac{0.06}{CO_2} = 0.10$						Average of 1 hour per animal: $\frac{0.01}{CO_2} = 0.045$							
Respiratory quotient: $\frac{CO_2}{O_2} = 0.6$						Respiratory quotient: $\frac{CO_2}{O_2} = 0.22$							

The data presented in table 10 shows the differences in the O_2 consumption and the CO_2 elimination of *Bulimus* and *Cyclostoma*. With a few exceptions, the quantity of oxygen used by *Cyclostoma* is about one-half of that consumed by a *Bulimus* of nearly the same volume kept under the same environmental conditions and for the same period of time. The elimination of CO_2 is also smaller to the same extent in *Cyclostoma* than in *Bulimus*. Whether the latter condition is due to storage of CO_2 in the tissues of the mollusks, is not answered by the experiments. In this connection, it must be emphasized that the behavior

of the animals may have had some influence on the findings. Although active, well-fed *Cyclostoma* specimens were selected from the field or the terrarium for the experiments, it was noted that the mollusks remained inactive and retracted in the shell during the entire experiment (experiments 2, 3 and 4). Others were active for a few hours, and then rested in the shells for the remainder of the tests. Unquestionably, the moisture content of the air had some influence, but in experiments 6, 7 and 8 mollusks, which had been kept on wet filter paper, were transferred to the bell-shaped dilatation of the capillary respirometer. O. Hesse, who studied the respiratory exchange of *Helix pomatia*, obtained difference in the O_2 intake and the elimination of CO_2 . He attributes these fluctuations to the irregular activity of the mollusks.

Further analysis of the table shows that the respiratory quotient for *Cyclostoma elegans* is very low; with the exception of experiment 2, it is below 0.5. The quotient for *Bulimus*, on the other hand, is above 0.5. In order to discuss these facts, it is advisable to review briefly the available knowledge on the respiratory quotient in gastropode mollusks. Relatively few estimations of the gas exchange on mollusks have been published. The most important data are summarized in table 11.

TABLE 11
PUBLISHED DATA ON THE RESPIRATORY QUOTIENT OF GASTROPODA

Gastropoda	Author	Respiratory Quotient $CO_2 : O_2$
<i>Helix pomatia</i>	Vernon.....	0.74 - 0.79
<i>Helix pomatia</i> active.....	O. Hesse.....	0.59 - 0.74 (Temperature 13.4 - 15.6 C.)
<i>Helix pomatia</i> inactive.....	O. Hesse.....	0.64 - 1.0 (Temperature 13.0 - 16.6 C.)
<i>Limax agrestis</i>	T. Thunberg.....	0.9 - 1.0
<i>Bulimus detritus</i>	K. F. Meyer.....	0.6

In applying the figures presented in table 11 to the discussion, it is advisable to recall that they have not been secured under comparable experimental conditions. However, it is evident that the quotient of *Cyclostoma* is the lowest thus far observed. Experiments 6 and 7, dealing with active mollusks kept under carefully controlled environmental conditions, are particularly significant. The quotient fluctuates between 0.36 and 0.39. No doubt these results are not attributable to the methods employed, or to the behavior of the mollusks, but they indicate either an incomplete oxidation or some other abnormal metabolic process of unknown nature. One recalls in this connection the low respiratory quotients during hibernation and the opinion expressed by Nagai that incomplete oxidations are in the main responsible for the

low quotient. Without adequate studies on the nitrogen metabolism of *Cyclostoma*, it is impossible to state that this explanation is the correct one. Furthermore, it must be borne in mind that the mollusks used in the tests on gas exchange were active summer animals in a humid environment. Although they remained quietly in their shell when kept on dry sand, these periods of inactivity even at high outside temperatures are by no means comparable with true hibernation. In this connection, it is recalled that M. Bellion and later W. Kühn expressed the view that hibernation is a specific physiological condition, which has probably very little in common with the ordinary periods of rest. In their studies, the respiratory quotient was found regularly lowered during hibernation. However, O. Hesse, who failed to detect changes in the respiratory quotient of *Helix*, is of the opinion that merely quantitative differences in the metabolism reduce the rate of the respiratory exchange during hibernation. The question naturally arises, "What are the metabolic processes which induce the low respiratory quotient?" Concerning them one is absolutely in the dark. Weinland has pointed out that the terrestrial gastropods possess the ability to store in their body various substances, as for example glycogen, perhaps fat and other unknown compounds. This peculiar function renders them more independent of the environment. It is generally admitted that certain pulmonata and prosobranchiata need oxygen to burn the large amounts of carbohydrates demonstrated in their tissues; however, exceptions (*Aplysia*, *Opisthobranchiata*, see Röhmann) have been observed, and it is not unlikely that catabolism of proteins, perhaps incomplete, occurs more frequently in certain mollusks than is indicated from the data, thus far available.

An attempt can now be made to explain the function of the organ. Although this study has established a number of new facts and has conclusively proved that the concretion deposits cannot be considered as parts of a true gland, the present knowledge is still inadequate to venture an exhaustive discussion of their physiological significance. A number of interpretations have been advanced by the various workers who have investigated the "glande à concrétion." Barfurth, who failed to find uric acid in the nephridium of *Cyclostoma*, expresses the view that the concretion deposits serve as storage places for waste products, in place of the inactive emunctories. The function would be similar to the peculiar excretophoric organ of the heteropod, *Carinaria mediterranea*, described by Fahringer. Furthermore, Barfurth emphasizes a striking histochemical similarity between the nephridium of *Cyclostoma*

and that of mussels. According to his studies, neither uric acid nor guanine or xanthine is found in the kidney of the two mollusks. In contrast to these contentions, it has been demonstrated by Perrier, Garnault and myself that the nephridium of *Cyclostoma* possesses definitive excretory functions; in fact, purine bases have been shown to exist in the extract of the organ. The conception of Barfurth can, therefore, be dismissed as untenable.

In his careful anatomical study of *Cyclostoma*, Garnault (p. 59) considers the concretion deposits as an organ for storage of uric acid which has been accumulated during periods of secretory inactivity of the nephridium. He writes concerning this subject as follows: "La glande à concrétions paraît être une glande vasculaire acquise chez les ancêtres des Cyclostomes, à l'époque où, quittant la vie aquatique, ils se sont adaptés à la vie terrestre. Les fréquentes périodes de repos qui sont imposées à ces animaux par les saisons, pendant l'hiver comme pendant l'été, et qui entravent la fonction de leur rein pendant des mois entiers, ont dû nécessiter la présence de cet organe de réserve, dans lequel s'accumule l'acide urique destiné à être résorbé pendant les périodes d'activité. Que devient cet acide urique? Il est probablement transformé en d'autres produits d'excrétion qui doivent être éliminés par le rein."

The first portion of Garnault's interpretation is not entirely supported by facts. It is recalled in this connection that the concretion deposits are usually smaller in spring after hibernation than in autumn after a period of marked activity. Mercier (p. 38) and Strohl (p. 594) cite a number of examples taken from the publications of Cuénot, in which it is shown that the evacuation of the excreta from the nephridium takes places at great intervals. The pulmonata, for example, discharge the nephridial content at intervals of from 15 to 30 days, and the aquatic *Anadonta* and *Unio* may eliminate colored excretions for a period of 130 to 140 days after an injection of a carmine solution. Furthermore, according to Krahelska and others, the nephridium of pulmonata can adapt its activity to the seasonal changes. In the light of these facts, it is certainly not necessary to assume that *Cyclostoma* requires an organ to store the products of excretion. In case a relationship exists between the nephridium and the concretion deposits, it is more plausible to believe that the latter serves in an auxiliary capacity to the former during the period of great activity and stimulated metabolism. Such a view is unquestionably expressed in the second part of the interpretation given by Garnault. The concretion deposits act as tem-

porary storage places or "excreta depots" for certain products of metabolism. The studies reported in this paper support this view, although the reasons for this storage of apparent useless waste material during periods of great activity remains undetermined. It has been pointed out that during starvation the accumulated purine bases may be synthesized and reemployed by the animal in his anabolism. Furthermore, Garnault and later Mercier believe that the concretion deposits do not retain their products, but dispose of them through deliquation and phagocytosis. Whether the "symbiotic" bacteria assist in this process of elimination has not been answered by the careful studies of Mercier. There are some indications that *Cyclostoma* lacking uricolytic enzymes in its tissues may be dependent on the ferments of certain bacteria to convert the concretions into products which are readily absorbed and perhaps later eliminated. In a previous chapter the resorption of the concretions by the amoebocytes has been described. The histological findings indicated a diphasic process; the resorption of the purine bases is followed by the ingestion of the organic substratum through amoebocytes. Mercier (p. 34) says in this connection: "la présence dans les dissociations de grosses concrétions naturellement transparentes, fait déjà constaté par Garnault, permet de supposer que les humeurs du Cyclostome renferment également une uricooxydase susceptible de solubiliser l'acide urique des concrétions." In fact, he expresses the view that probably the amoebocytes participate in the secretion of the "uricooxydase." While searching the recent literature on the enzymes of purine metabolism, so ably summarized by W. Jones, H. G. Wells and W. C. Rose, for evidence to support this view, it was found that one mollusk, *Sycotypus canaliculatus*, had been studied by Mendel and Wells for the presence of purine-transforming enzymes. Although their observations indicated the absence of an uricolytic ferment in mollusks, it appeared advisable to conduct a few experiments with *Cyclostoma*. These tests failed to demonstrate the presence of uricolytic enzymes in the tissues of *Cyclostoma elegans*. Unfortunately, no information was secured regarding the presence or absence of xantho-oxidase. A search for the presence or absence of xantho-oxidase appears very important in order to decide the origin of the uric acid in the concretion deposits. In mammals, this enzyme oxidizes xanthine into uric acid, which is believed to be the end product of purine metabolism. However, another source of uric acid has been observed in birds. Through the investigations of Minkowski and von Mach, evidence has been presented which indicates that synthesis of

uric acid from ammonia (urea) and lactic acid may occur. Just as in other species, the larger part of the uric acid excreted is an end product of protein metabolism. However, when the liver of the birds is extirpated, urea appears to be formed from amino-acids as in other animals, but instead of being excreted as such, it is transformed into uric acid. These investigations are exceedingly attractive and deserve application to the problems of uric acid excretion in mollusks, particularly in those species which are capable of storing uric acid in their mesodermal tissues. It is not unlikely that synthesis of uric acid from urea occurs under certain conditions, as this waste product has occasionally been demonstrated in the tissues of *Cyclostoma elegans*. In this connection it is also recalled that according to the careful studies of Sulima, synthesis of uric acid takes place in the digestive gland of *Aplysia* (nudibranch, marine Opisthobranch). One hundred grams of the moist organ may contain from 0.04-0.2 of pure, dry uric acid. As the intestinal content is free from purine bases, it is assumed by Sulima that the elimination of these waste products is performed by the emunctoria of *Aplysia*. On the other hand, it is known that certain mollusks excrete portions of their nitrogenous waste material by way of the intestinal tract. These and similar conditions deserve renewed investigation in connection with the analysis of the physiological factors responsible for the accumulation of purine substances in the connective tissue of the Cyclostomatidae.

Before closing this subject, the question of the anabolism of the purines should be considered. In a recent paper Rose (p. 560) tentatively suggested "that in conditions of physiological stress, such as starvation, the purines liberated in tissue wear and tear might possibly be reutilized for anabolic purposes." It is a matter of common experience that purines administered orally or parentally are not quantitatively recovered; however, the fate of the uneliminated portion is unknown. These observations are very suggestive, and may perhaps explain the storage of uric acid by *Cyclostoma*. It has already been indicated that the concretion deposits are smaller after a prolonged period of starvation or after hibernation. Anabolic synthesis of the purine substances present in the concretions may occur in view of the observations made on mammals. This phase of the exceedingly complex purin metabolism is open for further investigation. The finding of a *Cyclostoma sulcatum* with concretion deposits which were free from bacteria, and the demonstrations of large concretions in *Adamsiella* and *Chondropoma* species without bacteria indicate that probably either absorption or

elimination may take place independent of the intracellular bacteria. From the studies of Mendel and Wells and those just reported, it is reasonable to assume that other mollusks lack uricolytic enzymes in their organs, although as far as any one knows, they can handle uric acid without depending on the ferments of bacteria. All these speculative explanations are based on histological studies. Nothing definite is known regarding the factors which stimulate or suppress phagocytosis. The sections examined in this study fail to justify the general belief that the large concretions (type 3) develop on account of diminished or suppressed absorption, while the fine, band-like type 1 deposits are the outcome of intensive phagocytosis. Mercier has already pointed out that the phagocytosis of the purinocytes may represent merely a process to eliminate old and useless cells, a phenomenon quite frequently observed in the connective tissue of gastropoda. Besides freeing the tissues from unnecessary products of metabolism and perhaps controlling the multiplication of the bacteria, the phagocytosis may, according to Mercier who cites examples from Siedlecki and Gallaud, prevent losses and furnish the organism with useful substances resulting from the digestion of the purinocytes and the intracellular bacteria. This explanation supports the conception already advanced, namely, that the concretion deposits serve as temporary reservoirs for metabolic products which may be anabolized during enforced periods of starvation and inactivity. The existence of concretion deposits in several representative species of the family Cyclostomatidae and Annulariidae, operculate mollusks, which during dry seasons may starve for months without harmful results, even at tropical outside temperatures, is in this connection quite significant. Without extensive studies on the metabolism of *Cyclostoma*, one can merely conjecture by analogy deductions on the various chemical and morphological transformations which may take place. Moreover, nothing is known regarding the organs or tissues which may receive the products removed by the amoebocytes or the lymph and blood. Garnault and Mercier consider the concretions as waste products, and they therefore assume that the phagocytized material is eliminated by the nephridium or through the intestinal tract. The process is evidently more complex; the histological and physiological evidence is so incomplete that even speculative suggestions would be premature.

In his extensive review on the excretion in mollusks, Strohl (p. 526) writes as follows: "Ausser Harnsäure, Uraten und Guanin, lässt sich an Exkretions-produkten bei Schnecken kaum mehr etwas anführen.

. . . . Harnstoffuntersuchungen liegen keine vor." In the light of the biochemical tests which have shown that some of the bacteria found in the concretion deposits can convert uric acid to urea and ammonia, a renewed search for the carbamide, urea, in *Cyclostoma* appeared imperative. The new qualitative reagent for urea, xanthydrol, described by Fosse has been chosen. With the aid of this reagent, he demonstrated urea in the perivisceral liquor of a snail (escargot ?); he, furthermore, confirmed the well-known fact that urea is the main product of excretion in mussels, oysters, etc. Although the xanthydrol reagent has been primarily used by French workers (Marie and others) for the analysis of urine and blood, it is claimed to be particularly valuable for the demonstration of urea in tissues. During the last 8 years, a number of workers have reported their results. Policard was the first to attempt the histochemical demonstration of urea in tissues; he found the crystals in the collecting tubules of the injected mammalian kidneys. Chevallier and Chabanier, Stübel, Oliver, Walter, and Piras have employed xanthydrol to study the mechanism of urea excretion and have reported excellent comparative results. On the topographical distribution of the carbamide, Bonnet and Haushalter are the only workers who claim to have obtained irregular and inclusive results. They say (p. 397): "la teneur en urée des tissue autres que le rein et du sang, est si faible que le procédé au xanthydrol ne nous paraît pas assez sensible pour l'y déceler à l'état normal." It has been pointed out by Walter that the formation of the crystals depends largely on the solvent which is employed for the xanthydrol. The largest crystals, which could readily be detected in sections, developed at the site of the urea excretion when the tissues were injected with an ether-glacial-acetic-acid mixture saturated with xanthydrol. The microchemical xanthydrol test for urea in sections of tissues has thus far not been used on invertebrates.

A total of 135 nephridia of *Cyclostoma elegans* have been treated with xanthydrol-aceticacid-solution.* As a rule, 10 organs were fixed

* Technic: Xanthydrol cannot be secured in the market. It can be obtained from salol or xanthone as described by Oliver. I am indebted to Prof. Dr. P. Karrer of the Chemical Institute of the University of Zürich, who kindly furnished me with 200 gm. of the reagent, which had been prepared by one of his associates. The mixture, used for the fixing of the tissues, was prepared as follows: Two grams of xanthydrol were either dissolved in 30 c.c. glacial acetic acid (Stübel's method), or 2 gm. of the chemical were triturated with 10 c.c. of methyl alcohol, and 20 c.c. of acetic acid were added (Oliver's method). The preparation dissolved completely. It was, however, always filtered and was then a clear light yellow fluid. The nephridia of *Cyclostoma elegans* or pieces of the concretion deposits, the digestive gland and the intestines were fixed for 6 to 12 hours in the acetic-acid-xanthydrol solution. The tissues were then dehydrated in repeatedly changed absolute alcohol; passed through xylol and embedded in paraffin. Thin sections were mounted by means of egg-albumin on slides and stained with Mayer's hemalum.

in one bottle. In separate dishes, a slice of kidney removed from a mouse or rat which had been kept on a pure meat or lard and corn diet, was fixed on the same day and in the same solution.

These control specimens invariably revealed the dixanthyl-urea crystals not only lodged in the lumen of the tubules, but also in the cells of the glomeruli and the tubules. A careful search for crystals in the nephridia and the concretion deposits of *Cyclostoma* revealed one positive specimen. The mollusk had been collected in April at Liestal, two days before it was dissected and fixed in the xanthhydrol solution. Large rosettes of crystals were found in the nephridial cavity (fig. 30) or on the nephridial epithelium (fig. 31). Similar dixanthhydrol-urea crystals were lodged between the concretions and in the lymph spaces, but not in the "digestive" gland or the alimentary canal. They were never typically intracellular, although a casual inspection and even one photomicrograph (fig. 31) suggested such a position. Serial sections of the same *Cyclostoma* have been examined, and crystals have been observed in every field. Configuration, size, etc., leave no doubt that they are dixanthhydrol-urea crystals, indistinguishable from those seen in the kidneys and livers of mammals. However, the quantity of the crystals was considerably less in *Cyclostoma* than that seen in the rat or mouse.

Fifteen nephridia of *Cyclostoma sulcatum*, 12 of *Leonia mamillare* and 5 of *Helix pomatia* have been treated with xanthhydrol, but no crystals have been detected in the sections. The exceptional finding of urea in the body fluids of one *Cyclostoma elegans* hardly justifies the statement that this snail excretes occasionally urea aside from uric acid. In fact, one is inclined to attribute this observation to an accidental outside contamination. Fortunately, the complete record of this specimen, No. 69, is available. It was an active mollusk with a type 2 concretion deposit, which was very moist and contained culturally "fluorescens" and coli bacteria. A 6 per cent. xanthhydrol-acetic-acid solution, according to Stübel, had been used on this specimen. Subsequent series were fixed in the solution of Oliver (methyl alcohol-acetic acid). According to Walter, this solution permits of a rapid diffusion of urea and an escape of a considerable portion of the carbamide. Since the control kidney sections were all positive, although they never revealed the masses of crystals, as illustrated by Walter, it was not appreciated, until quite recently, that the negative findings in 149 *Cyclostoma* and 12 *Leonia* nephridia might probably be due to the technic employed. As Walter highly recommends the ether-acetic-acid solution saturated (75 per

cent.) with xanthydroxol according to Chevallier and Chabanier, it is contemplated to repeat the tests in the near future.

In connection with the description of the chemical studies on the extracts prepared from the concretion deposits, it was reported that traces of urea had been demonstrated. Similar positive results of Fosse with the extracts of other snails indicate that small amounts of urea are formed in the body fluid of Gastropoda or more specifically Cyclostoma. However, the amounts produced are so small that the relatively crude histochemical method with Oliver's xanthydroxol solution may not reveal them in their typical crystalline form. As already stated, further experiments must elucidate this point. In any case, sufficient reasons have been presented in connection with the recital of the bacteriological facts and the discussion of the purine metabolism that it would be rather surprising not to find urea in the body fluids of Cyclostoma. On the other hand, it is not unlikely that this substance is produced periodically under certain conditions, and is then rapidly synthesized to uric acid compounds. Until one knows the factors favoring urea formation or those responsible for the synthesis, the histochemical search for urea in Cyclostoma will remain an uncertain procedure, more dependent on chance than accurate information. In any case the method may furnish interesting data and the xanthydroxol reagent should be tried by other workers for a comparative study on the excretory products of mollusks.

The excretory function of the so-called purinocytes has been established in sections made through the concretion deposits of Cyclostoma sulcatum. A study of their evolution leaves no doubt that these cells continuously accumulate purine bases in the vacuoles of the cytoplasm or on the concretions. As the purinocytes are usually grouped around blood or lymph vessels, it is believed that they receive the products of metabolism through these channels. In this connection, the origin of the substances which are received and transformed by the purinocytes deserves some reflection. Are these substances either catabolic or anabolic products of metabolism? It is generally believed, although it has not been proved for the invertebrates, that the nucleic acids liberated in the disintegration of nucleoproteins of the tissues are hydrolyzed into their ultimate components in a manner quite similar to that which occurs in the case of exogenous nucleic acids during digestion in the alimentary tract. The source of the purines, whether in cellular catabolism or in absorption from the alimentary tract, plays no part in determining how oxidation shall occur. It is reasonable to assume that the purinocytes collect and store the various purine bases. In fact, they handle

merely the end products of the exogenous or endogenous purine catabolism. A number of facts speak in favor of this conception. For example, it has been noted that the cytoplasm of the purinocytes of *Chondropoma dentatum* may be reduced to a fine membrane, and yet the intracellular concretions apparently continue to grow. The purine bases present in the lymph or blood are merely precipitated on the primary and secondary centers, without the aid of the cellular functions of the purinocytes. On the other hand, it is not unlikely that the concretions are the result of anabolic processes. Synthesis of uric acid in the digestive gland of *Aplysia* has been reported by Sulima. As to this mechanism of purine formation, the literature affords very little information, and no final verdict can therefore be rendered. Irrespective of the ultimate origin of the substances which serve as precursors of the concretions, one cannot escape the impression that the mollusks retain and store from their catabolism end products, and this may under suitable conditions be returned to the synthetic metabolism. Mercier (p. 39) describes the purinocytes as "des véritables reins d'accumulation comparables, par exemple, aux cellules à urates du corps adipeux des Insectes." However, neither Garnault nor Mercier ventures any explanation regarding the origin of these cells. The organ as a whole is unquestionably of mesodermal origin. It is, probably, a manifestation of the highly specialized stage of the excretory function of the connective tissue elements frequently found in invertebrates. The view of Schaffer, that the mysterious purinocytes of *Cyclostoma* are analogous to the calciferous connective tissue cells found in the peri-intestinal tissues of *Bithynia* is disproved by the chemical tests, which show that purine bases and not calcium carbonate form the main portion of the concretions. Embryological studies alone may reveal the origin of the purinocytes, whether they are descendants of the cells of Leydig or modified endothelial elements. Functionally they lack phagocytic properties. They never carry pigment particles, nor do they ingest dyes or particulate matter. In connection with the description of the histological findings it is pointed out that the specific excretory function is strictly independent of the phagocytic activity exhibited by the connective tissue cells of other mollusks. Cuénot and later Mercier have shown that certain types of the excretory connective tissue cells of *Paludina vivipara* may contain crystals of hippuric acid, but invariably the same cells phagocytize particulate matter freely. Excretory and phagocytic function are, therefore, confined to one type of cell. The separation of the two functions, the excretory confined to the purino-

cytes, the phagocytic to the Leydig cells—as found in *Cyclostoma elegans*, *sulcatum* and *Leonia mamillare*, indicates for them a different origin than the plasma cells. The morphologist recalls the finding of two definite nephridial lobes in the prosobranch mollusks belonging to the Naticidae, Ampullariidae, etc., and he suspects the concretion deposits as the anterior, undifferentiated lobe of the left nephridium. In evaluating the recent studies of Sachwatkin, the comparative section material has been examined with the kind assistance of Prof. Dr. K. Hescheler. No evidence was found, which would confirm this view. The kidney of the adult Cyclostomatidae corresponds to the left nephridium of the Diotocardia; it is undivided and independent of the concretion deposits.

A search has been made repeatedly to secure *Cyclostoma* embryos, but without success. The smallest mollusk examined in this study measured about 3 mm., presented a well-defined and large concretion deposit. Barbieri (p. 282), who has studied the larval forms of these mollusks, reports the finding of concretions without stating the exact location or their mode of development. He writes as follows: “questi primi concrementi sono minuti, globosi, con un nucleo centrale più rifrangente di dimensioni variabili, edeposti, nel connettivo che circonda l'intestino posteriore.” The embryological differentiation of the purinocytes, which apparently occurs early in the development of the mollusk, remains to be determined.

The most important problem concerning the functional activity of the purinocytes presents itself in explaining the presence of bacteria in their cytoplasm. Garnault, Mercier and others considered it a phenomenon of symbiosis. This conception probably deserves revision, since it has been conclusively proved that individual mollusks (*Cyclostoma sulcatum*) may possess concretion deposits and yet no bacteria. In fact, another genus (*Chondropoma*) reveals concretion deposits free from micro-organisms. In some species of Cyclostomatidae, the cellular reactions toward the infecting bacteria may be slight, while in others, as for example *Leonia*, they may lead to definite connective tissue proliferation. In the light of the bacteriological findings recorded in this paper, it appeared to be of interest to scrutinize the intestinal tract of the mollusks which had noninfected purinocytes. Although bacteria were present in the intestinal content, nothing could be found in the epithelial lining of the mucosa which would explain the absence of micro-organisms in the peri-intestinal connective tissue. Again this fact is not surprising. Our knowledge concerning the function of the

intestinal mucosa, and especially its epithelium covering, is so incomplete that Macallum considers it one of the "great problems in pathology." Until one knows more about the conditions which permit the passage of bacteria or their toxins through the epithelium, it is deemed advisable to withhold even speculative suggestions.

Assuming that the gram-negative bacteria are present in the tissues, the question "How do these bacteria reach the cells?" deserves some consideration. Histological studies fail to indicate whether the microbes are chemotactively attracted and enter actively into the cytoplasm of the purinocytes or whether they are phagocytized. Observations on sections of *Cyclostoma sulcatum* and occasionally of *Cyclostoma elegans* and *Leonia mamillare* suggest an infection by contiguity of young cells intimately in contact with those already harboring clusters of bacteria. The finer mechanism and factors guiding this process remain, however, unknown. While considering the cultural findings made on the concretion deposits of *Cyclostoma elegans*, the ultimate origin of the intracellular bacteria of the purinocytes has been discussed. The hypothesis has been advanced that they are representatives of the intestinal flora, which have penetrated the mucosa and have been carried in the lymph or blood circulating in the deposits. This examination is supported by citing the observations of numerous workers (Wyssokowitch, Béco, Conradi, Coleman and Meyer, Masson and Regaud and others) on mammals. Bacteria and spores frequently penetrate the intestinal mucosa and are deposited in the regional lymph nodes, where they are phagocytized and remain latent for days, even months. Rous and Jones have reported interesting experiments, which enabled them to conclude that living tissue cells (endothelial cells, wandering cells, probably not fibroblasts) can protect ingested organisms from the action of destructive substances in the surrounding fluid.

Direct as well as indirect evidence favors the conception that each generation of the mollusk becomes infected anew by intimate contact with mollusks living in the same environment or the same colony. It is not unlikely that in this manner highly adapted bacteria possessing perhaps organotropic properties for the purinocytes, may be perpetuated. There is no reason to suspect that a type of specific organotropism reported by Rosenow for streptococci, by Helmolz for *B. coli*, and by Meyer, Shaw and Fleischner for the bacteria of the *Brucella* group, may not also occur in invertebrates. Highly specialized adaptation will unquestionably lead to tissue, or even cellular tropism, and the symbiotes will exhibit this property by invading either the excretory cells, the fat

body or the luminescent organs. The recent literature, on the other hand, cites in increasing numbers instances in which the micro-organisms are transmitted hereditarily through the egg to the next generation. This mode of transmission may take place, according to Buchner, in different ways, which have no particular interest in this discussion. It has been previously stated that an unsuccessful search has been made for embryos. Sections of the ovarium of two specimens of *Cyclostoma elegans* and one of *Leonia mamillare* stained by the Giemsa-Wolbach method have been carefully searched for the presence of bacteria in the ova. No micro-organisms were found. Fully developed eggs have, unfortunately, not been noted in the numerous sections examined. A final decision regarding the hereditary transmission of the *Cyclostoma* and *Leonia* cannot be rendered until suitable material has been studied microscopically and culturally. The remarks of Garnault (p. 124) "le passage dans les organes génitaux des oeufs sortis de l'ovaire est très rapide, . . . ils sont pondus immédiatement" deserves attention in this connection. Acquired infection early in the life of the mollusks appears more probable than transmission through the egg. The finding of an adult *Cyclostoma sulcatum* free from bacteria indicates that the infection is not transmitted hereditarily. From an experimental point of view, the absence of transmission through the eggs would offer great advantages. It would enable the biologist to rear the mollusks aseptically, just as Cohendy, Loeb and Northrup and others have succeeded in doing with other animals. The course of the infection and the part played by the bacteria could be studied experimentally and not merely by means of histological preparations and indirect analyses. The technical execution of such experiments is fraught with difficulties which cannot be surmounted until the life history and the ethnology of the mollusks are more thoroughly understood.

In the preceding pages the intracellular bacteria have been designated as "symbiotes." The word is used more for the sake of convenience than as the result of an absolute conviction that the phenomenon is one of true "symbiosis." The term "symbiosis" as originally used by the botanist Ant. de Bary (1879) denotes a condition of conjoint life existing between different organisms, which are benefited by the partnership in a varying degree. As Nuttall has pointed out, the term "symbiote," strictly speaking, applies equally to the partners; it has, however, come to be used also in a restricted sense as meaning the microscopic member or members of the partnership in contradistinction to the physically larger partners, which are conveniently termed the

"hosts" in conformity with parasitological usage. From the experiments and the findings reported, it is easy to assume that the mollusks derive some benefit from the intracellular bacteria as anabolists or catabolists of metabolic waste products, but, what possible benefit can the micro-organism derive from the association? Instances are cited by several writers, although it has rarely been shown experimentally that the host or macroscopic partner does not thrive without the presence of its symbiotes. For example, the symbiosis of the *Turbellaria*, *Convoluta roscoffensis*, with algae (*Zoo-chlorella*) is absolutely necessary for both partners, according to Keeble and Gamble. The union is so complete that the two factors are unable to survive separation. Another well-known example is afforded by the presence of bacteroids in the nodules of leguminosae, the micro-organisms being capable of fixing atmospheric nitrogen and thereby rendering nitrogen available for the plant. It has been shown by Hellriegel and Willfahrt, Beijerinck (1888) and others (see Hiltner 1904-1906) that the growth of the plants is reduced to the zero point in the absence of *Bacterium* (*Rhizobium*) *radicola* in the soil. In both examples, the microscopic partners receive in exchange either water, CO_2 and metabolic waste products (*Convoluta*) or nutritive protein material (*Rhizobium*).

The recent literature on "symbiotes" has been carefully searched for other examples, but only theories have been found which are not established on any scientific basis. The function of the microscopic "symbiotes" and their benefit to the host are explained, but little or nothing is said regarding the possible advantages to the micro-organisms. Sulc believes that the yeasts in the fat bodies of aphids and coccids may decompose urates, while Pierantoni thinks that the symbiotes may produce an enzyme that aids in the digestion of sugars. Brues and Glaser suppose that the proteolytic and lipolytic enzyme of the *Pulvinaria*-symbiote assist in breaking down the adipose cells of the host. Xylophagous *Lepidoptera* (*Cossus*, *Nonagria*, *Sesia*, etc.) possess as symbiotes, according to Portier, intestinal fungi which may aid in the digestion of the otherwise nonassimilable cellulose. Peklo found in phytophagous *Hemiptera* different symbiotes, *saccharomycetes*, which may assist in the assimilation of the imbibed leaf sap rich in mineral substances, carbohydrates and glycosides, while coccoid organisms are interpreted as *Azotobacter chroococcum*, which may fix free nitrogen and supply the host with nitrogenous substances, thereby meeting a deficiency in its food supply. A number of investigators have even gone a step further, and advanced the theory that the host secures

protein material by finally digesting the symbiotic algae and fungi. Dangeard, for example, speaks of a direct nutrition ("une nutrition directe") of *Paramaecium bursaria* by the ingestion of the symbiotic zoochlorellae, while several botanists, Frank, Magnus and others, compare the "fungivorous" orchids with the insectivorous plants which secure their nitrogenous food by the digestion of protein material of insects. In this connection, it must be emphasized that digestion of fungi or zoochlorellae occurs independently of symbiosis. As for example in cases in which the auxiliary partner has become unnecessary or superfluous (see N. Bernard and Le Dantec 1892). Concerning *Cyclostoma*, Mercier believes that the mollusk by removing through phagocytosis the bacteria regains some of the substances, which it had previously furnished to the "symbiotes." He concludes that "le Bacille tire profit du Cyclostome," although he does not detail the various factors, which might prove this contention. Buchner's suggestion that the intracellular organisms are benefited by being protected within the hosts from the drastic atmospheric influences of heat, cold, desiccation, etc., is a trifle unreasonable. Furthermore, this view that the bacteria are also assured constant nourishment and opportunity to propagate is likewise not appealing.

If it can be proved that the micro-organisms cultivated from the concretion deposits are identical with the intracellular bacteria, it is difficult to believe that these organisms should manage to live and propagate better in the cells of animals than outside in nature. It must always be remembered that the host tissues or its body fluids produce powerful, inhibitive substances in order to hold the propagation of the micro-organisms within reasonable limits; otherwise the host would be killed. According to Cantacuzène, Chahovitch and others, such immune substances are produced by a number of invertebrates in response to a bacterial invasion. In the light of the numerous interesting studies by Erber on the agglutinatoric properties of the hemolymph of *Helix pomatia*, it will be necessary to study the serological behaviors of *Cyclostoma* lymph on the intracellular bacteria of the purinocytes. The numerical control of the bacteria by the host is very definite in the purinocytes of *Cyclostoma*. The presence of inhibitory forces is surely a handicap in the struggle for existence. The views of Mercier should therefore be reversed; namely, the host may receive some service from the bacteria in the form of enzymes which may during periods of starvation transform waste material into substances useful in the metabolism of the snail, while the micro-organisms, as far as it is evident,

derive no benefit from the association. It is not unlikely that they would secure more benefit as true parasites or disease producers. This thought leads to another question of importance. Is the "symbiotic," "communal life" observed in *Cyclostoma* an example of true parasitism or even of disease?

"Symbiosis" must be regarded as a condition of life balancing between two extremes—complete immunity and deadly infective disease (Nuttall). In fact, it has been shown by botanists, N. Bernard, Magrou and others, that the theories and the factors governing immunity from parasites or "symbiotes" are essentially the same. It is difficult to believe that symbiosis originated in any other manner than through a preliminary stage of parasitism on the part of one of the associated organisms. The struggle between the two partners leads in the course of time to a form of mutual adaptation. The symbiotic relationship of orchids and fungi experimentally investigated by N. Bernard, Burgeff and others, has been carefully studied from this point of view, but nothing similar has been done in "symbiosis" of animals. It is worth considering that such structures as the mycetocytes, bacteriocytes and mycetomes in the insects are a survival of previous profound pathologic changes, or, as N. Bernard has shown for the plants, tuberisation is the sequel or a symptom of an advanced adaptation of the plants to a communal life with fungi. During an early period, the bacteria, fungi, etc., now found as harmless entities within the insects, are probably parasites producing pathologic conditions and disease. Acquired immunity later becomes inherited, and the micro-organisms are gradually gotten under control. The insects do not rid themselves of the invaders on account of the fact that transmission from generation to generation is established with remarkable precision. Still later, the invaders lost all of their harmful effects, and since they secrete enzymes that prove serviceable to the hosts, the conflict ends in mutual adaptation; just as in plants (orchids and their mycorrhizas) the symbiosis between the tissues of the host and the micro-organisms is a phenomenon of parasitism, infection or disease, which has finally become essential to the existence of the animal. The knowledge concerning symbiosis represents a chapter in the pathology of bacterial and parasitic infections which may help to advance the understanding of such phenomena as local immunity or tumor formation due to parasites (Fibiger and others).

Considered from this general biological point of view, it is interesting to reflect concerning what would have happened to those animals

which harbor intracellular organisms if they had never been invaded by the parasites. It is impossible to imagine that such structures as the mycetocytes and mycetomes with their parasites could have developed without altering the physiology and morphology and consequently the habits of the hosts. The "symbiotic" processes are obviously concerned in the evolution of certain species of animals and plants. Whether such a condition plays a rôle in the Prosobranch family of Cyclostomatidae, deserves consideration in the future.

The "symbiotic" complex in the purinocytes deserves attention from another point of view. Cultural studies have indicated the occurrence of three to four groups of gram-negative bacilli in the concretion deposits. Although it has by no means been proved that the cultivated bacteria are identical with the microscopically demonstrable organisms, it is of value to consider the significance of this tri- or quadri-symbiotic process. According to Pinoy (1913), the bacteroids of the root nodules of a leguminous plant are myxobacteria, *Chondromyces crocatus*. It is found essential for successful cultivation of the micro-organisms apart from its host plant or in vitro, that it should be grown in association with a species of micrococcus (related to *Micrococcus luteus*). This association is highly specific and cannot be replaced by any other bacterial species; the micro-organisms are mutually interdependent for their growth. Biochemical studies conducted with the various types of bacteria isolated from *Cyclostoma* suggest that at least two of the organisms may be mutually dependent on their products of metabolism. It is, therefore, not unlikely that a superimposed "symbiosis" may be necessary to maintain the original monosymbiotic invasion.

In conclusion, the findings can be summarized as follows: The concretion deposits of *Cyclostoma elegans*, *lutetianum*, perhaps *sulcatum*, *mauretanicum*, *Olivieri*, *Leonia mamillare* and *Tudorella ferruginea* serve as temporary storage places or reservoirs for the products of metabolism which may be anabolized or catabolized during enforced periods of starvation and inactivity. Furthermore, it is not unlikely that "symbiosis" with intracellular bacteria may aid in the transformative processes and as such be of benefit directly or indirectly to the animals. By studying mollusks belonging to the Annulariidae it has become evident that the supposed "symbiosis" deserves, however, further investigation. Certain Algerian snails reveal definite signs of reaction in form of connective tissue proliferation, etc. Moreover, individual mollusks which are free

from intracellular bacteria have been found. In fact, it is quite evident that the purinocytes may develop their concretions independently of micro-organisms. The bacteria are probably not "symbiotes" in the true sense of the word, but parasites. Finally, it is hoped that this investigation may serve as an outline for further work on the physiological aspect of the concretion deposits.

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PLATE 1

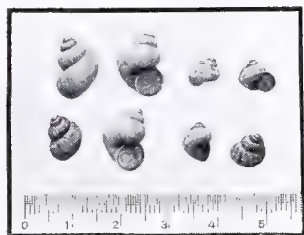


FIG 1

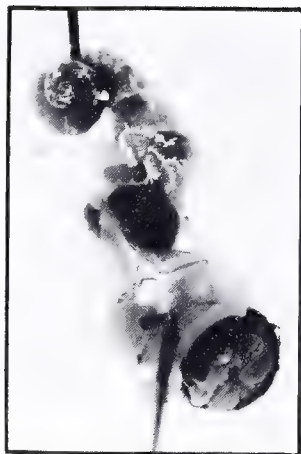


FIG 2



FIG. 3



FIG. 4

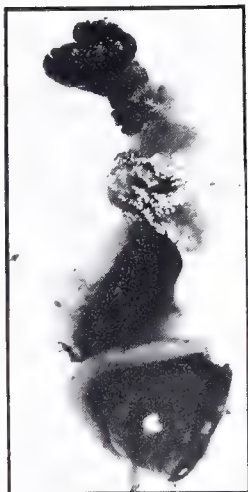


FIG 5



FIG 6

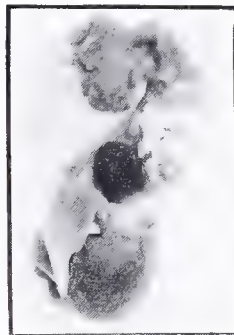


FIG 7

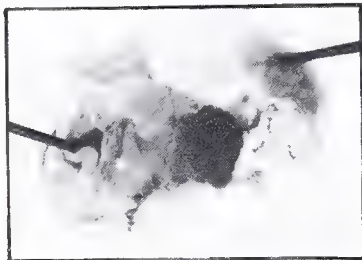


FIG 8

CYCLOSTOMA ELEGANS DRAP (Origin: Liestal and Mendrisio, Switzerland)

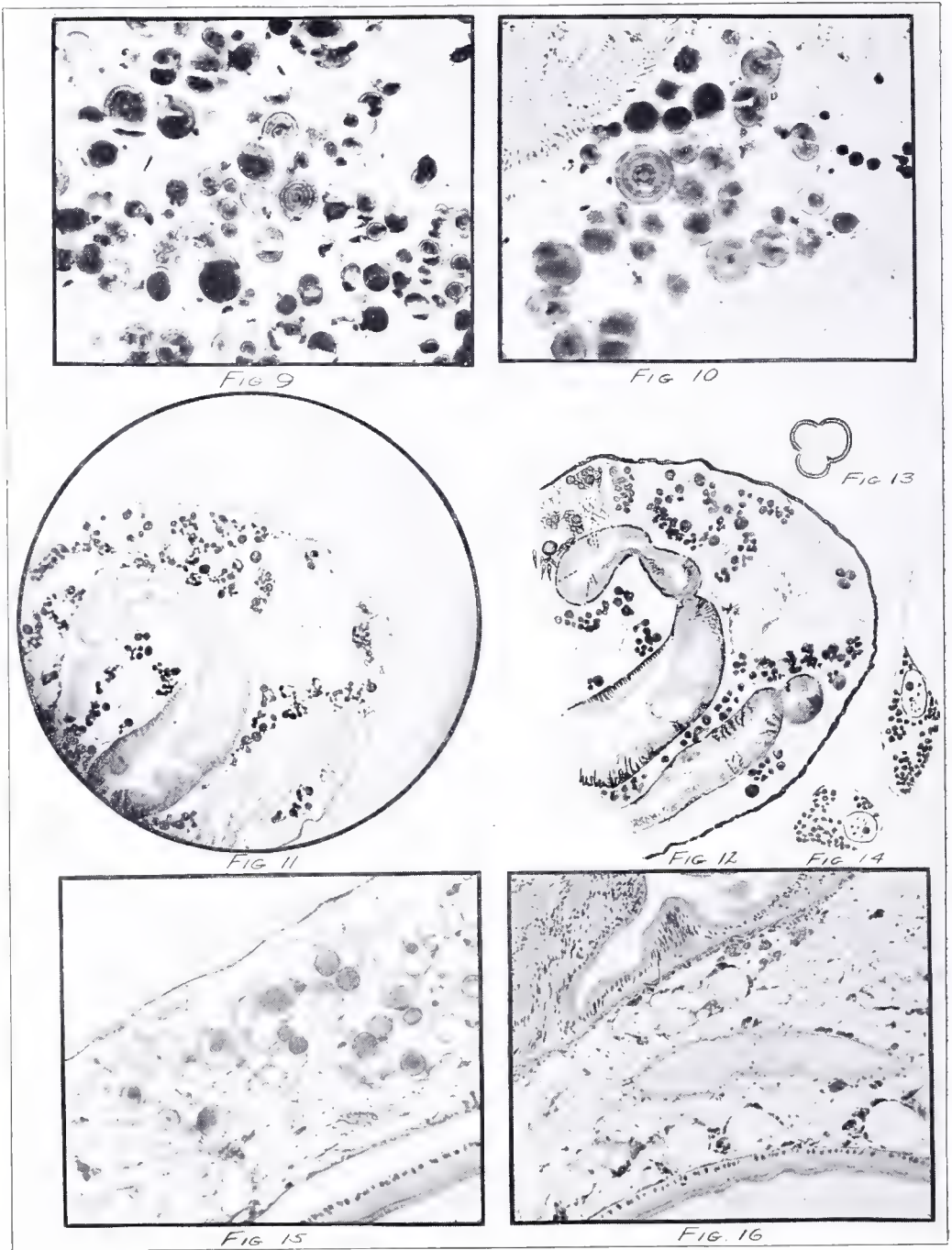
Fig. 1.—Living mollusks of different ages, reduced.

Fig. 2.—Cyclostoma without shell showing concretion deposits beginning near nephridium and surrounding the intestines; this type has been designated in the publication as "small," type 1; the succulent infiltration of the connective tissue is very distinct. Microluminar 3 X.

Figs. 3 and 5.—Concretion deposits of the type 2. Note the appearance of the connective tissue. Microluminar 3 X.

Figs. 4, 6, 7 and 8.—Concretion deposits of the type 3. The cement-like, chalky infiltration of the connective tissue is very striking. Microluminar 3 X.

PLATE 2



CYCLOSTOMA ELEGANS DRAP

(Origin Liestal and Mendrisio)

Corrosive Sublimate-acetic acid Mayer's Haemalum-Orange G.

- Figs. 9 and 10.—Concretions sectioned showing central nucleus and lamellation. $\times 75$.
 Figs. 11 and 12.—General distribution of the concretion deposits in the connective tissue surrounding the intestinal tube. $\times 20$.
 Fig. 13.—Drawing of concretions, partially dissolved with KOH. $\times 75$.
 Fig. 14.—Excretory cells ("nephrocytes") with brownish granular pigment bodies. $\times 650$.
 Fig. 15.—Appearance of concretions in the connective tissue, showing the central nucleus and uniform lamellation. $\times 75$.
 Fig. 16.—Hemolymph vessel surrounded by aggregations of concretions, partially dissolved by the staining process, showing phagocytosis. $\times 75$.

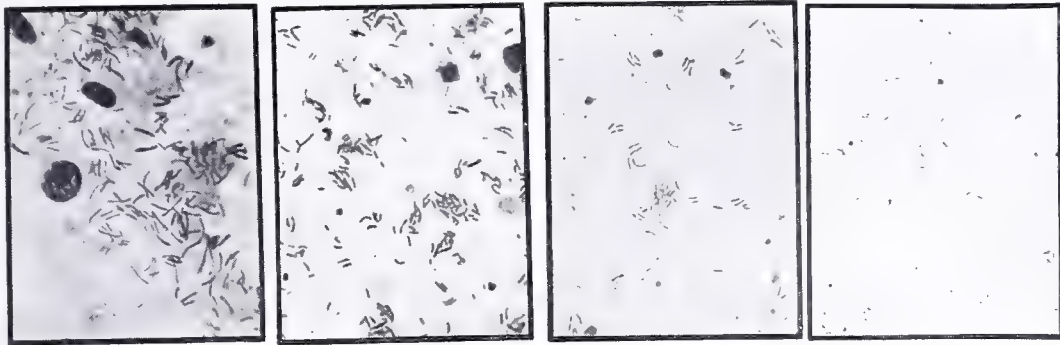


FIG 17

FIG. 18

FIG 19

FIG 20

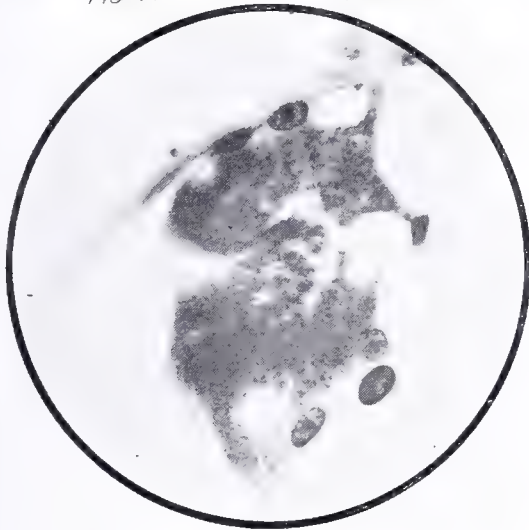


FIG. 21

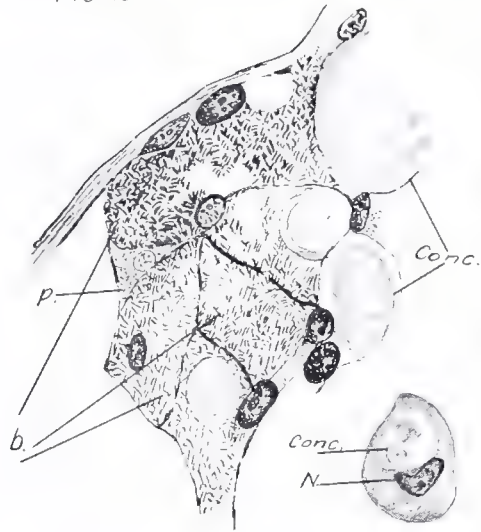


FIG 21A.

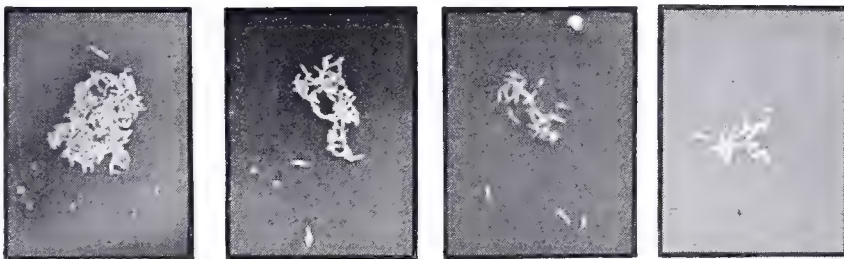


FIG 22.

FIG 23

FIG. 24

FIG 25

THE BACTERIA OF THE CONCRETION DEPOSITS OF CYCLOCSTOMA ELEGANS DRAP

Fig. 17.—Smear prepared from an active specimen (Grenoble, France). Moist fixation in Schaudinn's sublimate-alcohol. Giemsa-Wolbach stain. $\times 650$.

Fig. 18.—Smear prepared from an active specimen (Liestal) Giemsa-Wolbach stain. $\times 650$.

Fig. 19.—Smear prepared from a hibernating specimen (Mendrisio, Tessin); Alcohol-ether fixation; Gram stain; decolorized. $\times 650$.

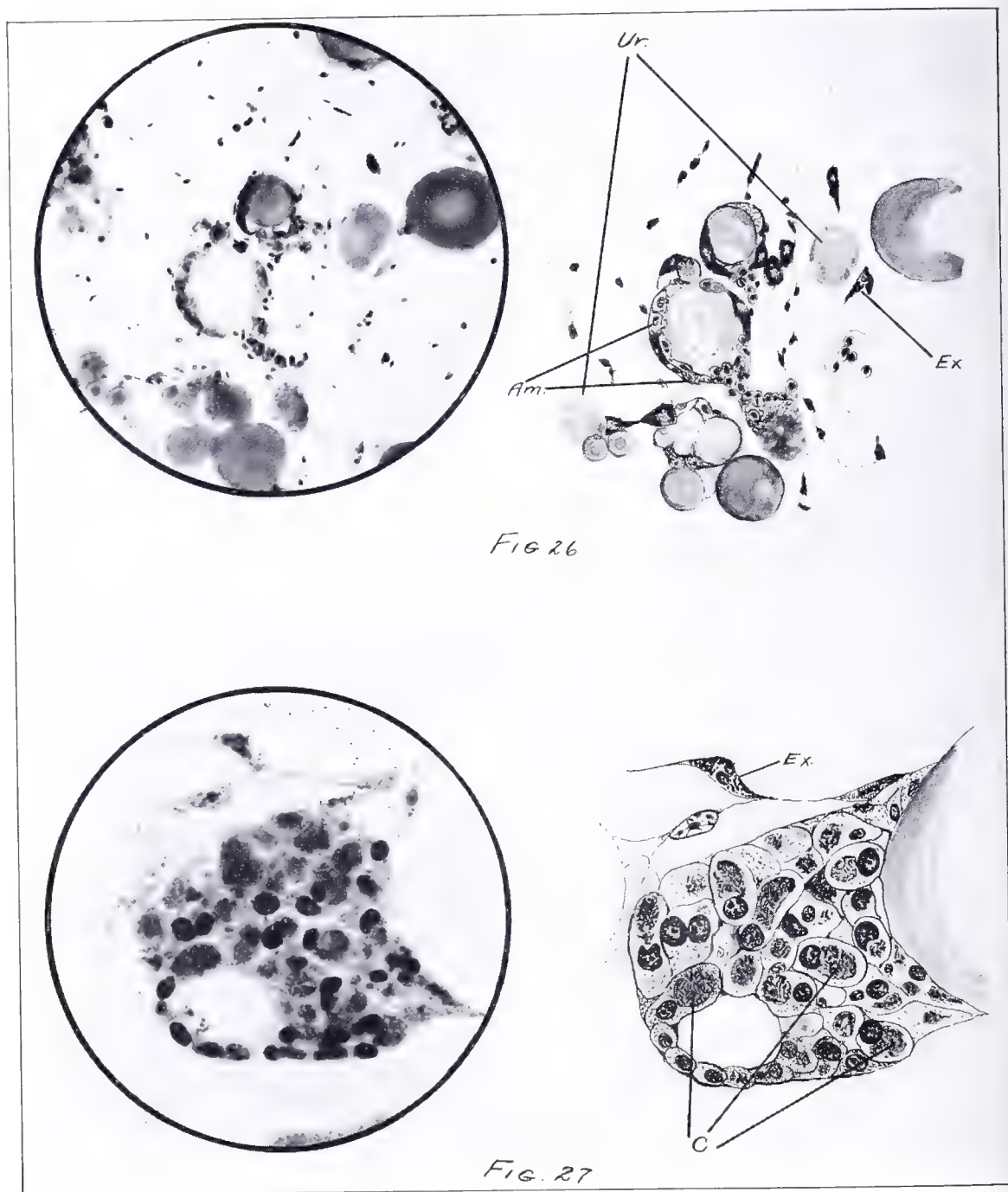
Fig. 20.—Smear prepared from a hibernating (December) specimen (Liestal); distinctly granular staining and diphtheroid in appearance; heat fixation; Manson's methylene blue. $\times 650$.

Fig. 21.—A group of purinocytes showing in part the intracellular arrangement of the concretions and bacteria. Concretions partially dissolved by the staining process. Schaudinn's sublimate-alcohol. Giemsa-Wolbach stain. $\times 650$. *p* indicates young purinocytes without bacteria and concretions; *b*, bacteria; *conc.*, concretions.

Fig. 21A.—Drawing of an isolated young purinocyte with a vacuole containing several small concretions; *conc.* and *N* indicate an irregular nucleus.

Figs. 22, 23, 24 and 25.—Smears prepared with "Cyanochin" according to Eisenberg; origin: 22 and 23, Liestal active forms, 24, Grenoble active form, 25, Marseille hibernating form. $\times 650$.

PLATE 4



PHAGOCYTOSIS IN THE CONCRETION DEPOSITS OF
CYCLOSTOMA ELEGANS DRAP
(Active April-form, Liestal)
Corrosive sublimate-acetic acid-fixation

Fig. 26.—Concretions surrounded and partially attacked by amoebocytes. Excretory cells with pigment granules. *ur* indicates partially uricolyzed concretions; *am*, amoebocytes; *ex*, nephrocytes. Dilute toluidine-blue solution. $\times 75$.

Fig. 27.—An area composed of phagocytic cells, a large number of them containing agglutinated and partially digested clumps of bacteria. *C* indicates concretions dissolved; *ex*, nephrocyte with pigment granules. Giemsa-Wolbach stain. $\times 650$.

PLATE 5

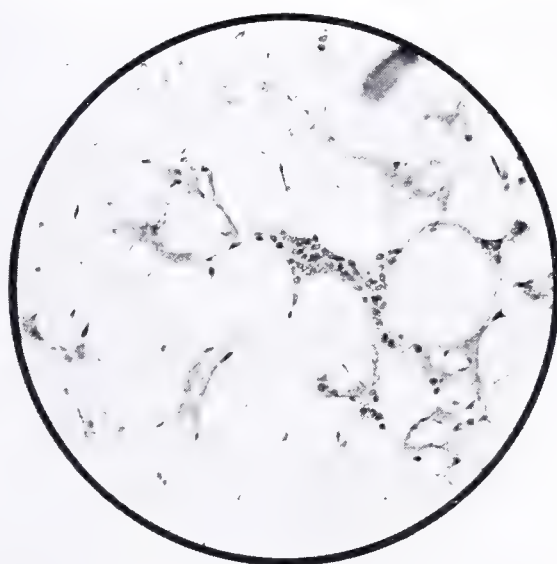


FIG. 28

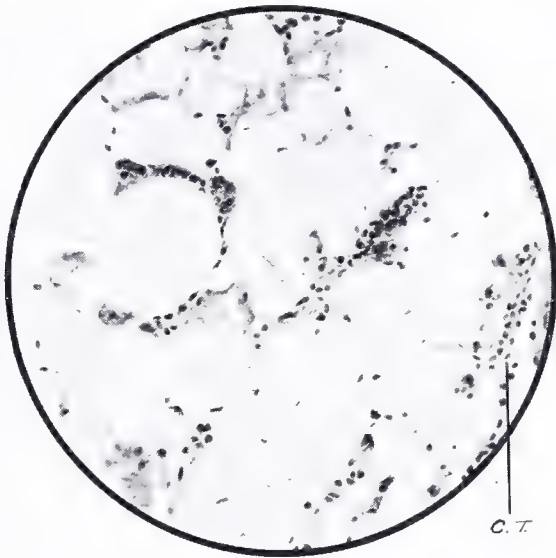


FIG. 29

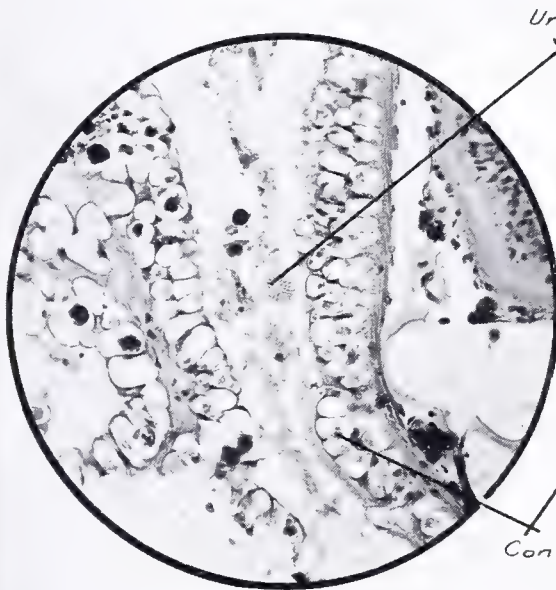


FIG. 30.

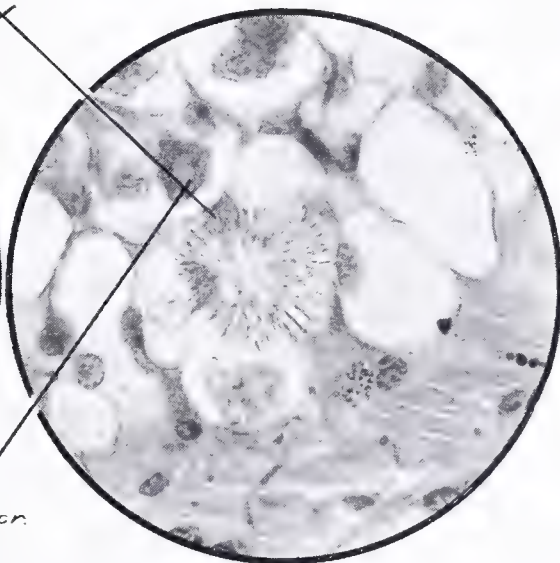


FIG 31

CYCLOSTOMA ELEGANS DRAP

Figs. 28 and 29.—Areas composed of amoebocytes surrounding the concretions and uric acid cells. Corrosive sublimate-acetic acid. Giemsa-Wolbach stain. $\times 75$. Fig. 28, *Cyclostoma* from Liestal (April specimen); fig. 29, *Cyclostoma* from Marseilles (May specimen). The concretions are partially dissolved by the staining process. C.T. indicates scar tissue formed by amoebocytes.

Fig. 30.—Nephridium with a rosette of urea-xanthidrol crystals: Ur-X, in the lumen; *concr.* indicates nephridial concretions. Liestal active spring form. Methyl-alcohol-xanthidrol-acetic-acid, hemalum. $\times 75$.

Fig. 31.—Group of vesicular excretory cells of the nephridium with a rosette of urea-xanthidrol crystals starting from the center of a vacuole. Clumps of brownish concretions in the other vacuoles. Same mullusk as preparation of fig. 30, treated in the same manner. $\times 650$.

PLATE 6

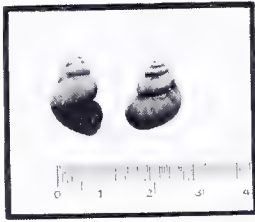


Fig 32

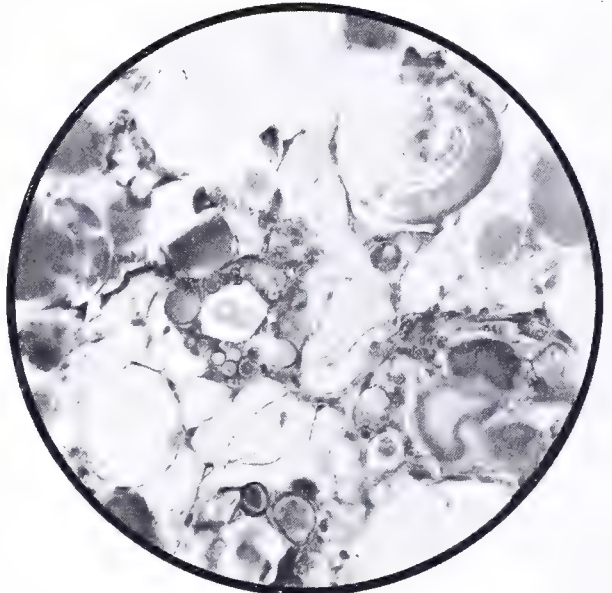


FIG 33



FIG. 34

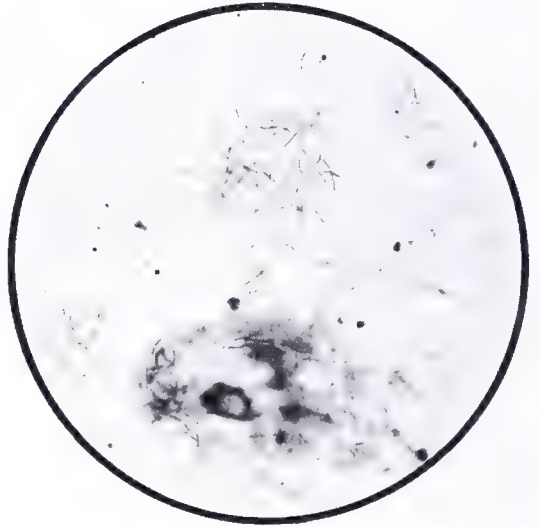


FIG 35

CYCLOSTOMA LUTETIANUM BOURGUIGNAT

From Forêt de la Sainte Baume
(Courtesy of Mr. E. Margier, Nîmes)

Fig. 32.—Living specimens, reduced.

Fig. 33.—Section through concretion deposits. Note large concretions surrounded by clusters of small crystalline concretions inside of urinocytes cells. Zenker's fluid; hemalum; orange G. $\times 150$.

Figs. 34 and 35.—Smears prepared from purinocytes showing long and slender bacteria. Two different mollusks—moist fixation in Scaudinn's sublimate-alcohol. Giemsa-Wolbach stain. $\times 650$.

PLATE 7

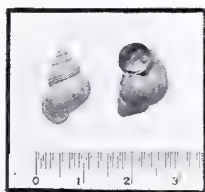


FIG. 36

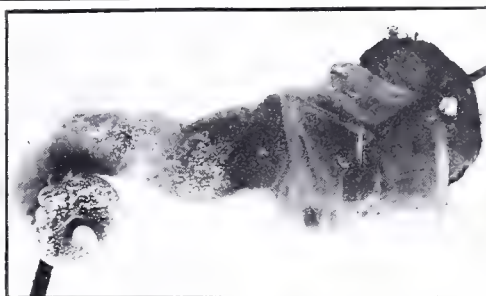


FIG. 37

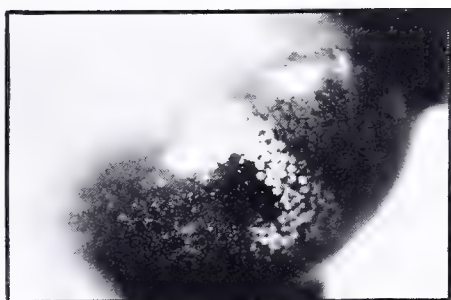


FIG. 39

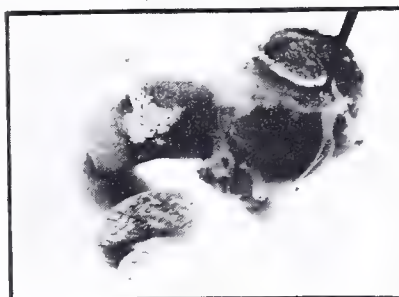


FIG. 38

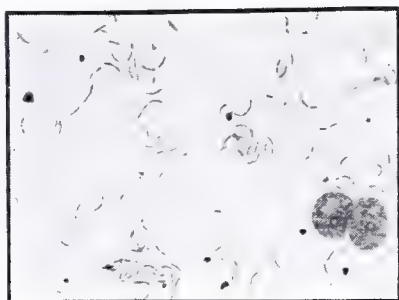


FIG. 40

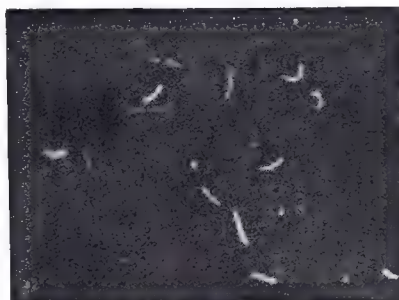


FIG. 41

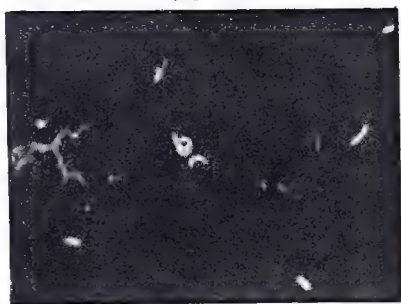


FIG. 42

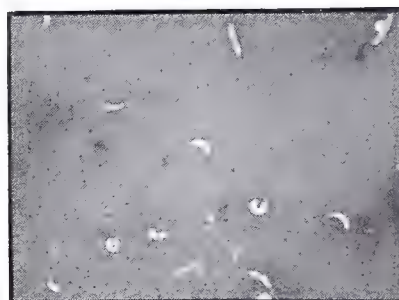


FIG. 43

CYCLOSTOMA SULCATUM DRAP

From Bandol and Marseilles

(Courtesy of E. Margier, Nimes and Prof. Vayssière, Marseilles)

Fig. 36.—Living specimens from Nimes—reduced.

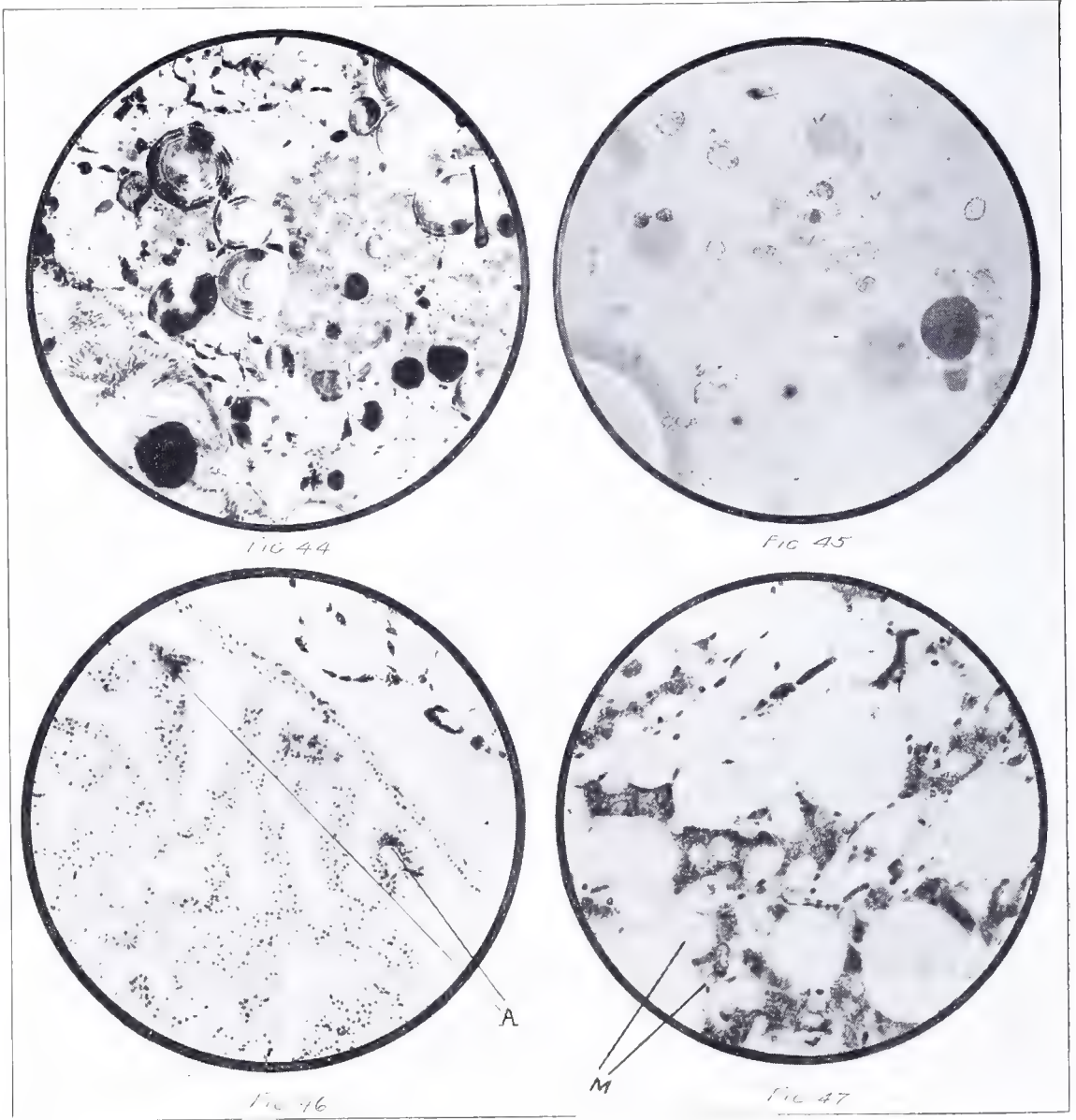
Figs. 37 and 38.—Mollusks without shell showing concretion deposits. Micro-luminar (16 mm.). $\times 3$.

Fig. 39.—A portion of the concretion deposit shown in fig. 38 enlarged. Note the size of the concretions. Microsummar (42 mm.). $\times 9$.

Fig. 40.—Smear prepared from purinocytes showing rather thick and curved bacilli. Corrosive sublimate-alcohol fixation and Giemsa-Wolbach stain. $\times 650$.

Figs. 41, 42 and 43.—Smears prepared with "Cyanochin" according to Eisenberg. Note the faint inside staining in some of the bacilli, of figs. 41 and 43. $\times 650$.

PLATE 8



CYCLOSTOMA SULCATUM DRAP

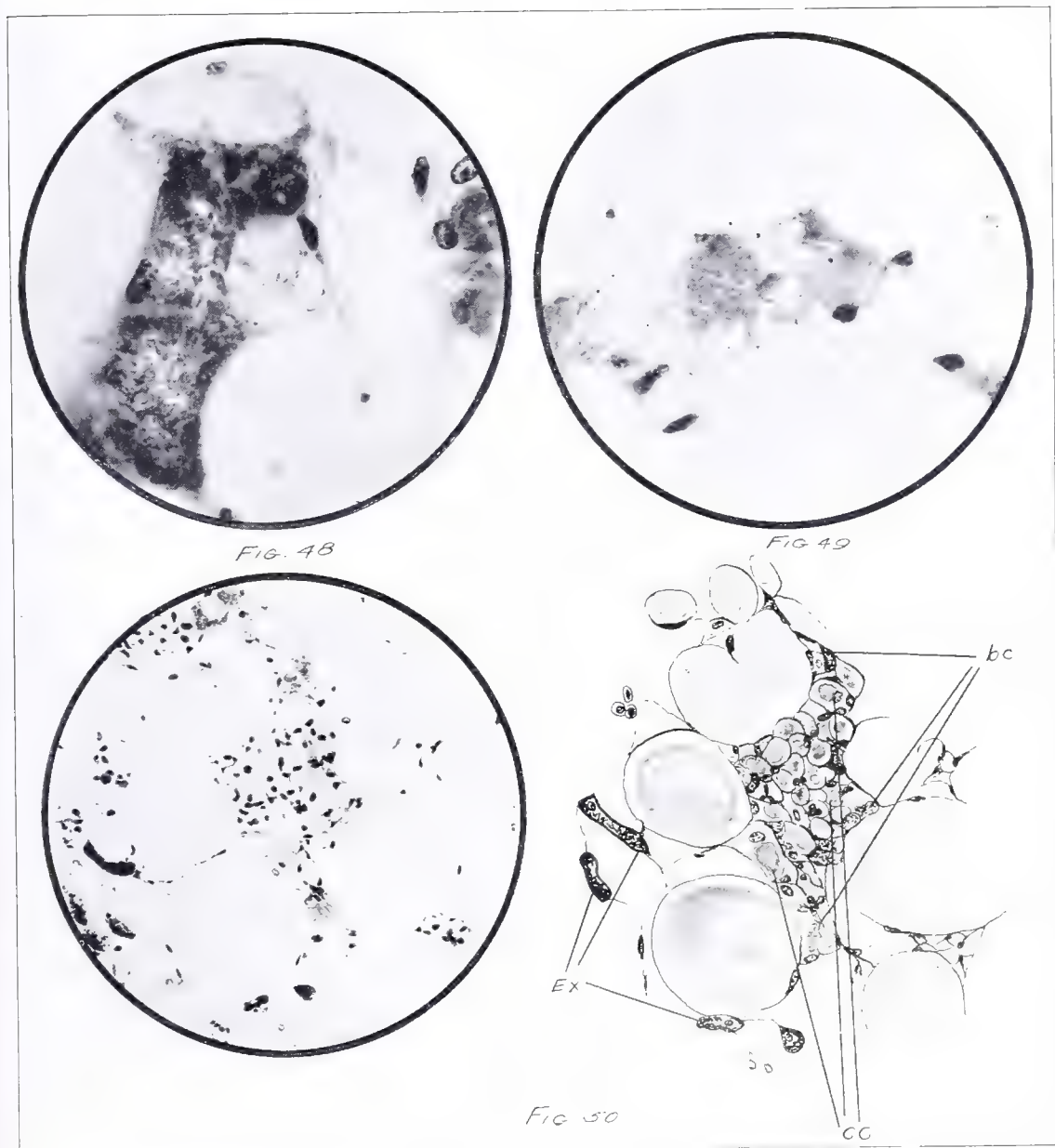
Fig. 44.—Section through the concretion deposits; large concretions and areas consisting of purinocytes with small transparent concretions. Zenker's fluid; hemalum; orange G. $\times 75$.

Fig. 45.—Unstained teased preparation of concretion deposits—large opaque and small transparent concretions. $\times 75$.

Fig. 46.—Section through the nephridium. Note the absence of concretions in the vacuoles of the nephridial cells; *a*, amoebocytes.

Fig. 47.—Section through the concretion deposit illustrating the purinocytes filled with clusters of bacteria and small concretions. Note the faintly stained matrix (*M*) of the large partially dissolved concretions. Corrosive sublimate, acetic-acid. Giemsa-Wolbach stain, $\times 150$.

PLATE 9



CYCLOSTOMA SULCATUM DRAP

Corrosive sublimate-acetic acid—Giemsa-Wolbach stain

Fig. 48.—Purinocyte with intracellular bacteria. Concretions dissolved. $\times 650$.

Fig. 49.—Two purinocytes—one (left) with long filamentous bacteria, and another (right) with an eosinophilic cytoplasm containing a small concretion, but no bacteria. $\times 650$.

Fig. 50.—An island of purinocytes embedded in the connective tissue surrounded by large (partially dissolved) concretions and composed of group of cells free from bacteria each containing a vacuole with a concretion (cc). Some cells (bc) contain a few filamentous faintly stained bacteria. Ex indicates nephrocytes. $\times 150$.

PLATE 10

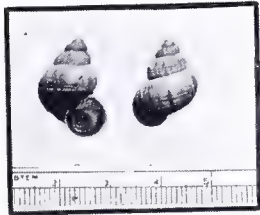


Fig. 76



Fig. 77

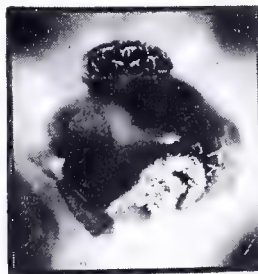


Fig. 78

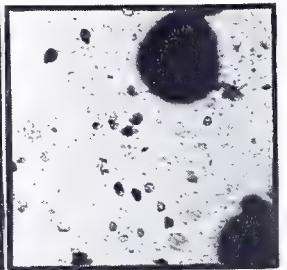


Fig. 79

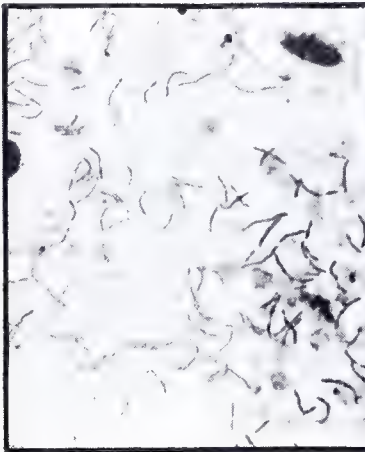


Fig. 80

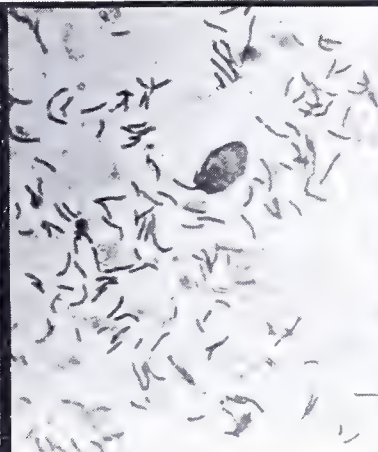


Fig. 81

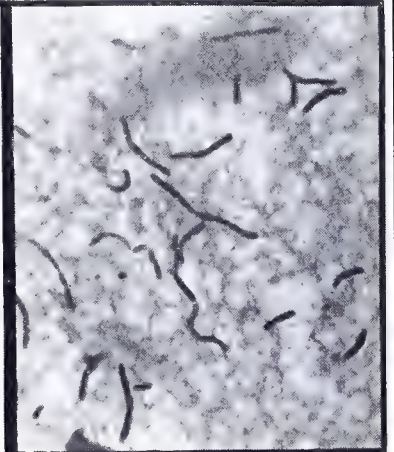


Fig. 82

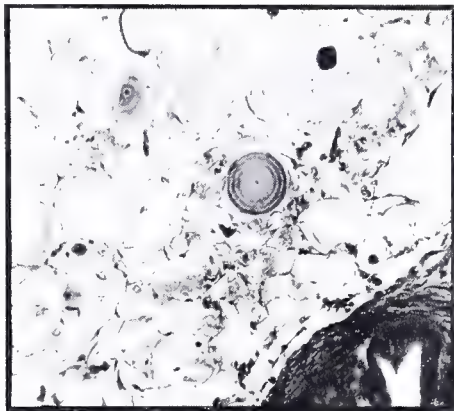


Fig. 83

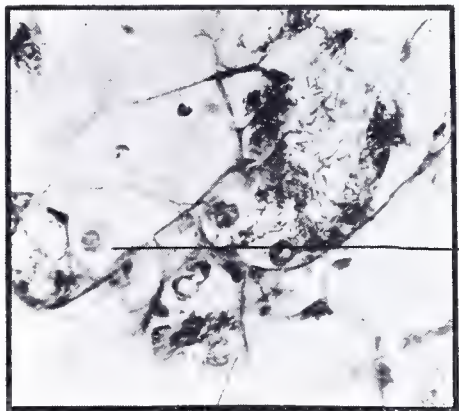


Fig. 84.

CYCLOSTOMA MAURETANICUM PLRY

From Rar el Maden, Beni-Saf

(Courtesy of Mr. M. P. Pallary, Algeria)

Figs. 76, 77, and 78.—Living specimen, natural size. Mollusk without shell showing large brown-whitish concretion deposits surrounding the intestines. $\times 2$ (77) and $\times 3$ (78).

Fig. 79.—Unstained teased preparation, showing two types, small and large, opaque and transparent, amorphous concretions. $\times 75$.

Fig. 80.—Smear prepared from purinocytes; bacteria large; curved Gram stain. $\times 650$.

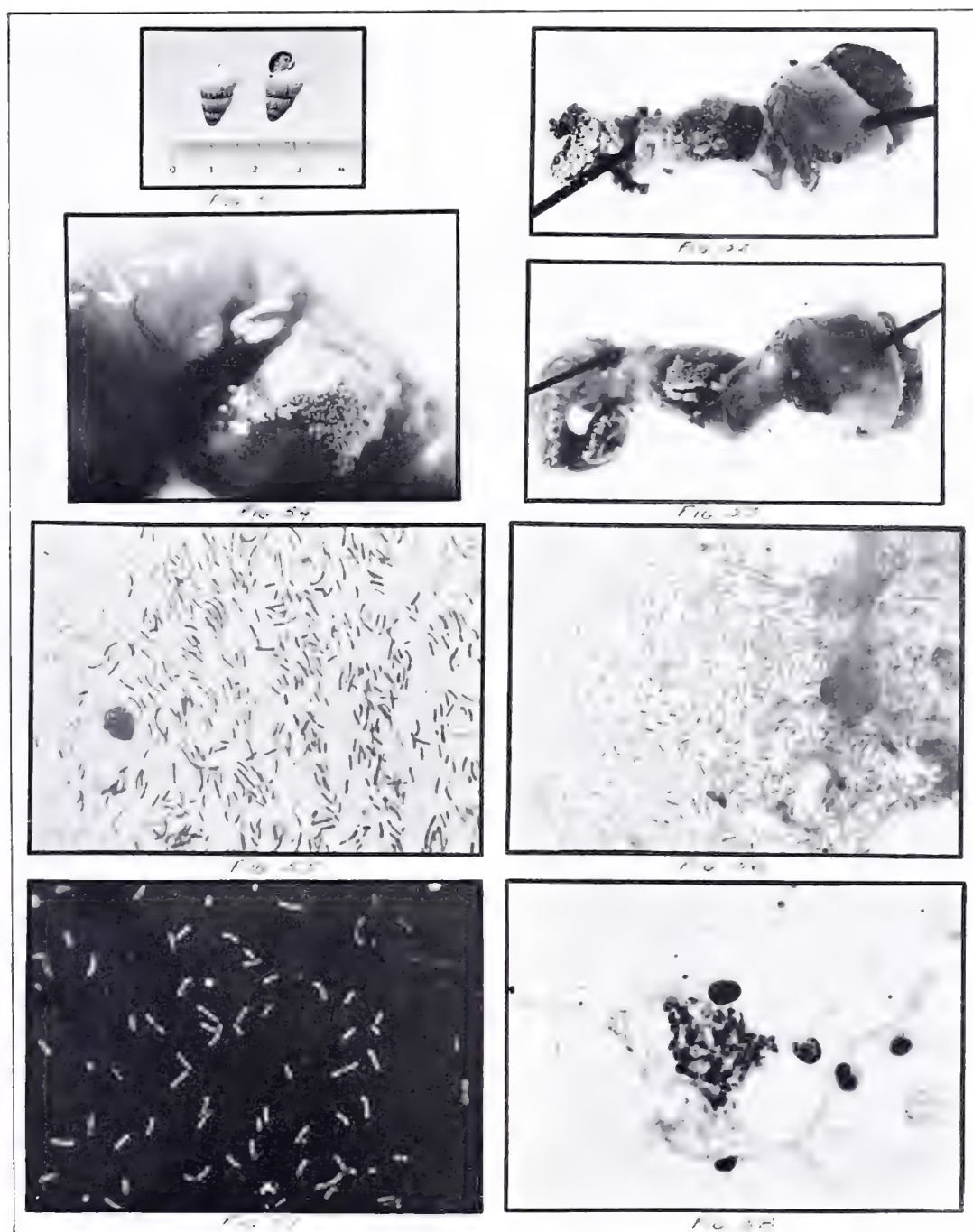
Fig. 81.—Smear from different specimen; methyl-alcohol Manson stain. $\times 650$.

Fig. 82.—Smear from same specimen as fig. 81—moist fixation in corrosive sublimate-alcohol; Giemsa-Wolbach stain. $\times 650$.

Fig. 83.—Section through a concretion deposit; islands of purinocytes with small concretions surrounding the large lamellated partially dissolved concretions. Iron-hematoxylin stain. $\times 75$.

Fig. 84.—Purinocytes with small partially dissolved concretions (F) and intracellular bacteria. Giemsa-Wolbach stain. $\times 150$.

PLATE 11



LEONIA MAMILLARE LINK

From Oran-Eckmuhl, Algeria
(Courtesy of Mr. M. P. Pallary)

- Fig. 51.—Living specimen, reduced.
 Figs. 52 and 53.—Mollusks without shell showing shieldlike concretion deposits surrounding the posterior portion of the intestines. Microluminar, 16 mm. $\times 3$.
 Fig. 54.—A portion of the concretion deposit shown in fig. 53, enlarged. Note the size of the whitish concretions. Microsummar 42 mm. $\times 9$.
 Fig. 55.—Smear prepared from purinocytes—bacteria large and thick. Corrosive sublimate. Giemsa-Wolbach stain. $\times 650$.
 Fig. 56.—Smear of the same material as shown in fig. 55 fixed with methyl alcohol and stained with dilute Manson's blue. Note uneven staining. $\times 650$.
 Fig. 57.—Smear prepared with "Cyanochin." $\times 650$.
 Fig. 58.—Purinocytes with intracellular bacteria. Corrosive sublimate. Giemsa-Wolbach stain. $\times 650$.

PLATE 12



FIG. 59

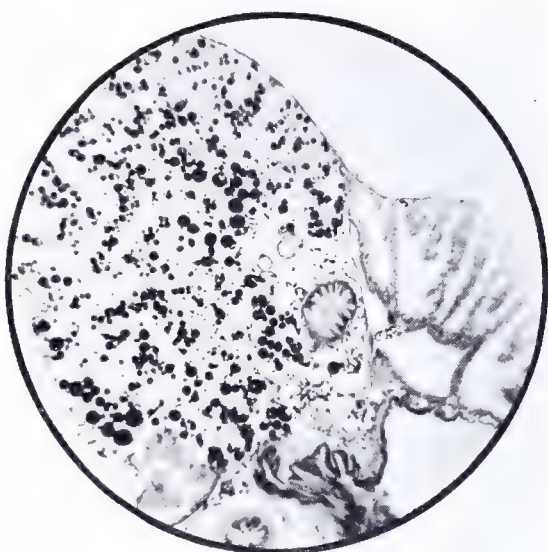


FIG. 60

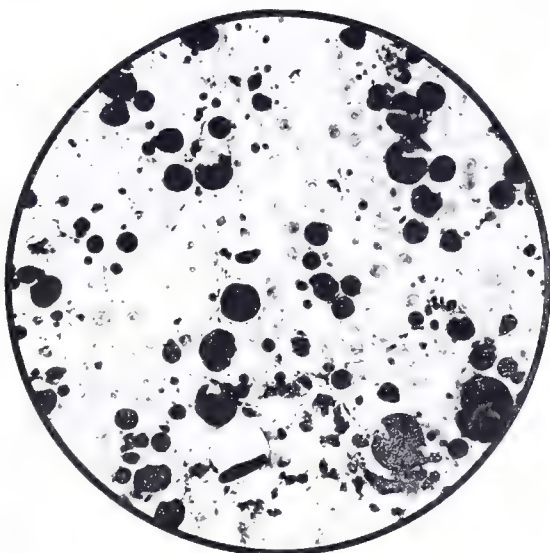


FIG. 61

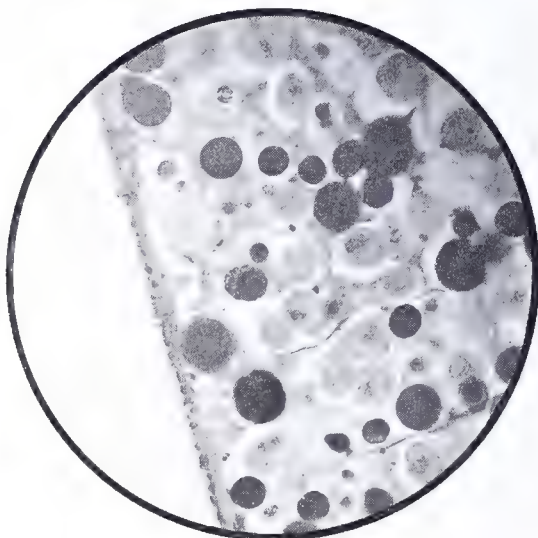
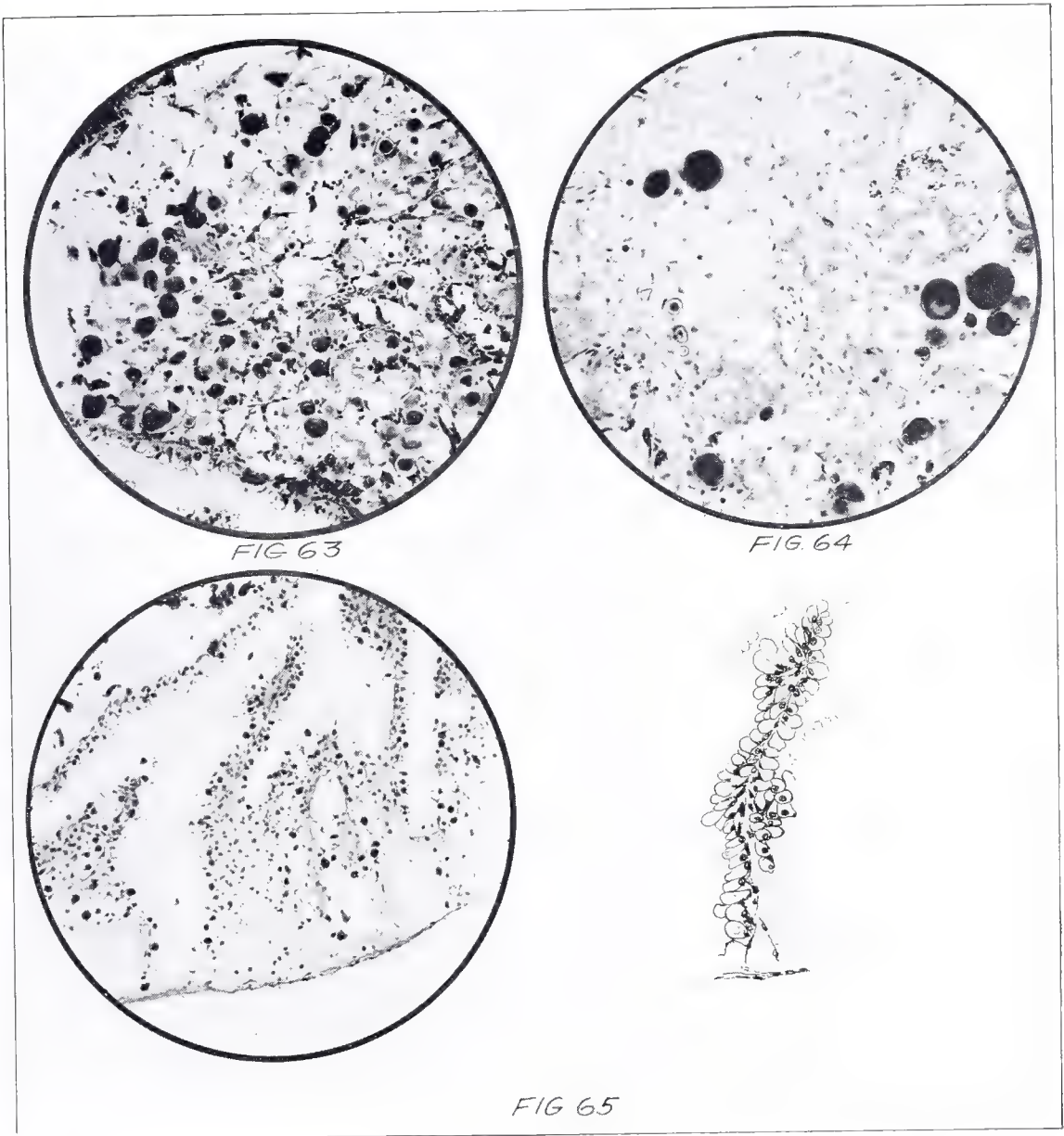


FIG. 62

LEONIA MAMILLARE LINK

Fig. 59.—Unstained, teased preparation showing two types of concretion—opaque and transparent, with opaque primary center.
 acetic acid; hemalum; orange G (60 and 61). Note the predominance of transparent concretions. $\times 20$. Note the bands of dense connective tissue forming definite septums throughout the concretion deposits. Fig. 61, concretions partially dissolved. $\times 75$. Fig. 62, dilute
 Figs. 60, 61 and 62.—Sections through the "shield" of concretions. Corrosive sublimate-Manson's blue. $\times 150$.

PLATE 13



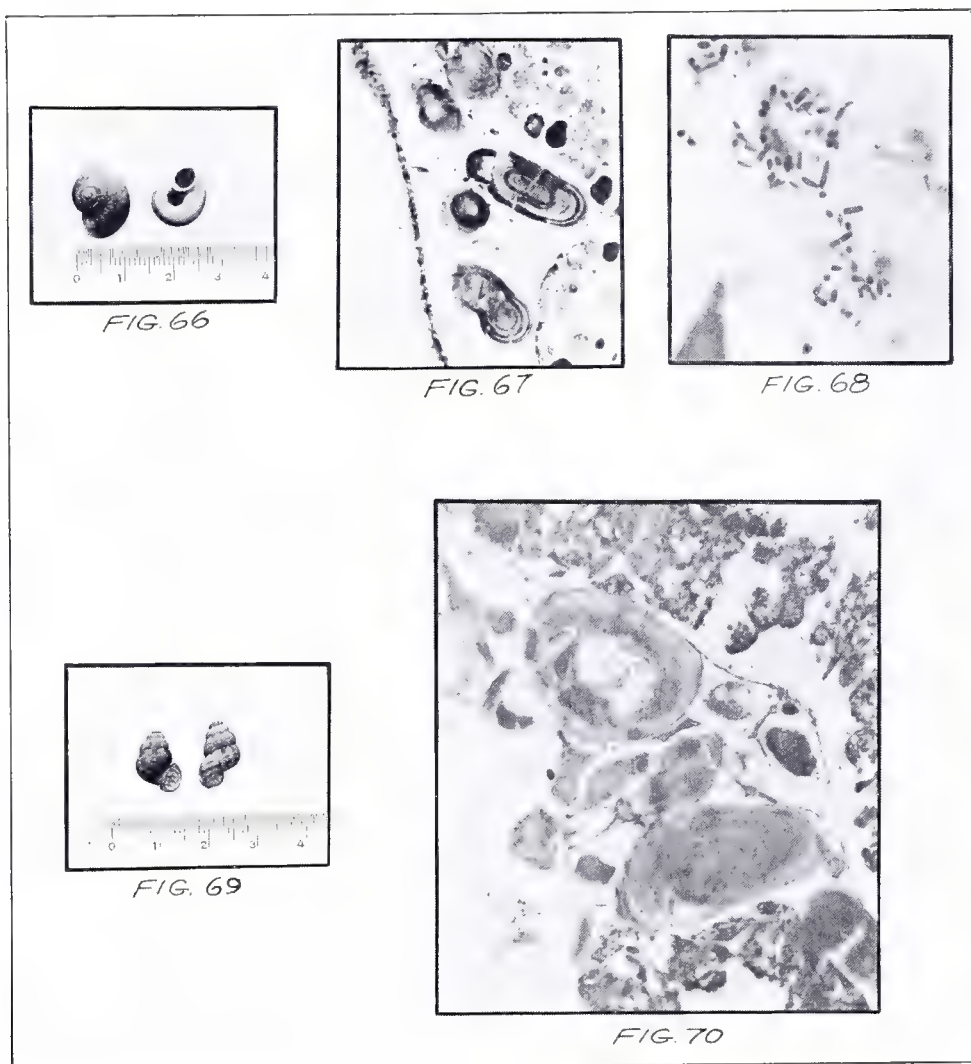
LEONIA MAMILLARE LINK

Fig. 63.—Connective tissue proliferation and formation of septums—different mollusk, as shown in fig. 62. Corrosive sublimate, acetic acid; hemalum; orange G. $\times 75$.

Fig. 64.—Focus composed of cicatricial tissue separating clusters of transparent concretions. Section from the same specimen as shown in fig. 63. $\times 150$.

Fig. 65.—Section through the nephridium. Lamellae with excretory epithelial cells; greenish concretions in the vacuoles. Note single layer of cells. Corrosive sublimate; acetic acid; Giemsa-Wolbach stain. $\times 75$.

PLATE 14



TUDORELLATA PUTRE (G) PFR.

Guantanamo Bay—Eastern Cuba
From the United States National Museum
(Courtesy of Dr. Paul Bartsch)

Fig. 66.—Specimens preserved in alcohol; natural size.

Fig. 67.—Large oval concretions situated between digestive gland. Hemalum. $\times 75$.

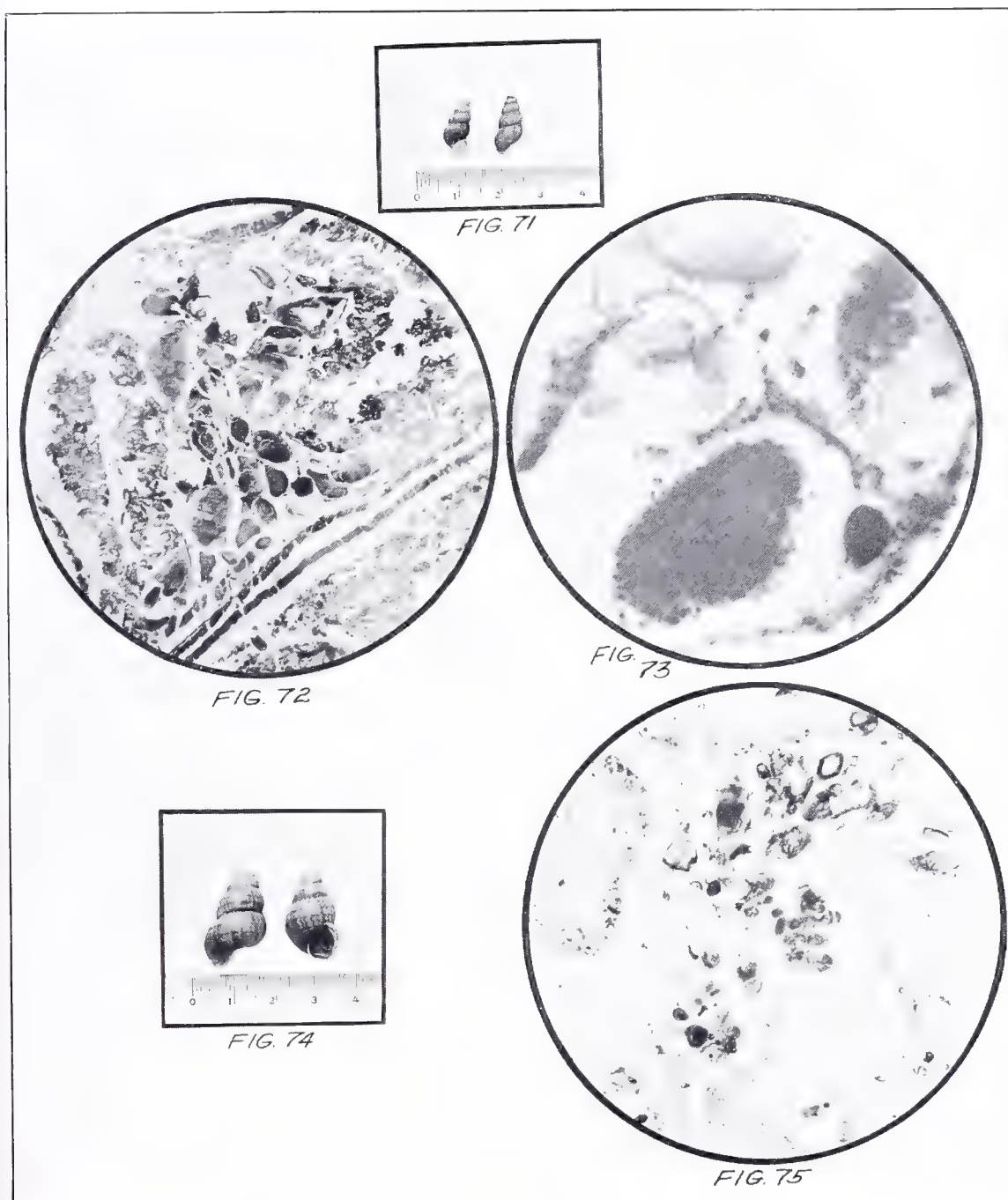
Fig. 68.—Bacteria found near concretions. Section stained with Manson's blue. $\times 650$.

ADAMSIELLA VARIABILIS C. B. AD.

Hollymount, Jamaica
From the United States National Museum

Fig. 69.—Specimen preserved in alcohol; natural size.

Fig. 70.—Large concretions and remnants of purinocytes. Material preserved in alcohol. Hemalum. $\times 150$.



CHONDROPOMA SUBRETICULATUM (MALTZ)

From Haiti

Received from the United States National Museum

Fig. 71.—Specimens preserved in alcohol; natural size.

Fig. 72.—Section through concretion deposits. Note oval shape of concretions and peculiar clusters of round bodies resembling “yeasts.” Stained with dilute carbol-fuchsin. $\times 75$.

Fig. 73.—One of the clusters at greater magnification. $\times 650$.

CHONDROPOMA MAJUSCULUS, PFR.

From Cuba

Received from the United States National Museum

Fig. 74.—Specimen preserved in alcohol.

Fig. 75.—Section through concretion deposits; specimen poorly preserved. Hemalum. $\times 75$.

PLATE 16



Fig 85



Fig. 86



Fig. 87

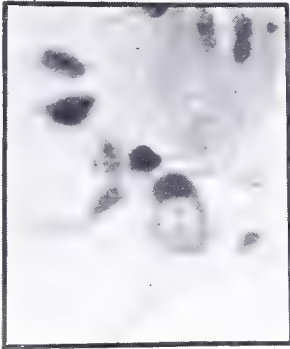


Fig. 88

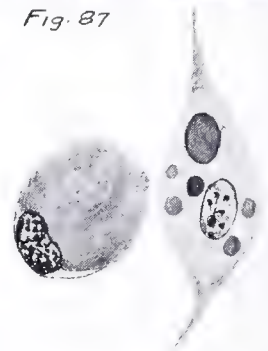
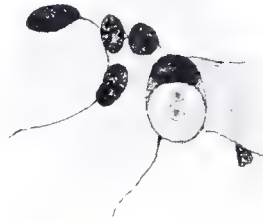


Fig 89

Fig 90

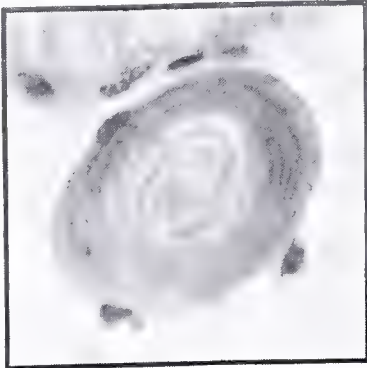


Fig 91



Fig. 92

CHONDROPOMA DENTATUM SAY.

From Florida, U. S. A.
(Courtesy of Mr. C. T. Simpson)

- Fig. 85.—Mollusk without shell showing whitish concretions extending from the nephridium (black) to the tip of the last coil. $\times 3, 1$.
 Fig. 86.—Section through a portion of the mollusk; oval concretions in connective tissue surrounding the intestines. Iron-hematoxylin. $\times 75$.
 Fig. 87.—Section through the nephridium. Giemsa-Walbach. $\times 75$.
 Fig. 88.—Purinocyte with a small concretion and "primary centers." Iron-hematoxylin. $\times 650$.
 Fig. 89.—Drawing of a purinocyte with a lamellated and striated concretion. $\times 650$.
 Fig. 90.—Drawing of a glycogen-carrying Leydig cell. $\times 650$.
 Fig. 91.—Intracellular lamellated concretion; note the attached nucleus and the fine cellular membrane.
 Fig. 92.—Lamellated concretion located in the peri-intestinal connective tissue. Iron-hematoxylin. $\times 75$.

MICROBIC RESPIRATION

I. THE COMPENSATION MANOMETER AND OTHER MEANS FOR THE STUDY OF MICROBIC RESPIRATION

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Summary

INTRODUCTION

In the current methods of studying organisms, stress is placed on the medium, its composition and reaction, and on the chemical changes which it undergoes. The oxygen requirements, as expressed by Pasteur's¹ terms, aerobe and anaerobe, are supplied somewhat mechanically, and little or no attention is given to the gaseous products of cell activity unless the organism happens to evolve considerable quantities of gas. The result is the misleading classification of organisms into aerogenic or gas-producing, and nonaerogenic or no gas producers. The truth is that all living organisms are actively engaged in gas production.

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¹ Compt. rend. Acad. Sc., 1863, 56, p. 1192.

Cell respiration is a necessary phenomenon of life possibly more tangible than any other chemical change induced by a cell. It is to be looked on, not as a side reaction, but rather as the source of the energy which is needed to vitalize the protoplasm. Just as the combustion of fuel is necessary to produce the energy which will cause a machine to do work, just so energy must be generated within the cell to enable it to function. Many organisms, without doubt, are able to produce this growth energy by the partial combustion of the very material which furnishes, at the same time, the constituents or groups that are rebuilt into cellular matter or protoplasm. In other words, growth energy and growth matter may be supplied by one and the same food. On the other hand, it is probably equally true that some organisms require energy-producing substances which are entirely distinct from those which are utilized as cell builders. Thus, it is well known that glycerol is a necessary constituent which must be added to certain mediums in order that the tubercle bacillus may grow. It will be shown that, in this case, glycerol is a fuel and as such the source of the growth energy which enables the organism to utilize the other constituents of the medium as actual food or growth material.

The oxidation changes which take place within a unicellular organism, taken as a whole, are in no wise different from those of the constituent cells which go to make up the higher animal or plant. Carbonic acid is always the end-product in this combustion, and, in the case of aerobic organisms, it is in definite relation to the amount of oxygen consumed, that is to say, for a given medium the respiratory quotient is constant. Viewed only from one side, it is customary to state that such organisms require oxygen. This is true, but only in part, since there is good reason to believe that without carbonic acid such oxygen cannot be fully utilized by some organisms.

Carbonic acid is essential to the life of higher animals; its decrease by overventilation leads to startling and even serious results. Waste product though it is, it is indispensable as a stimulus to the respiratory center, and is thus of benefit to the body complex. It may therefore be laid down, as a working hypothesis, that carbonic acid is an equally essential stimulus for the growth of unicellular organisms. Evidence for this view is to be sought among the slow-growing organisms, but as yet it has not been produced.

It may be pointed out in this connection that a pathogenic germ, growing within the body, is under a constant though low oxygen and

carbonic acid tension. This fact is only too often overlooked, and every possible effort is directed at improving the culture medium. And yet, it is reasonable to believe that the initial cultivation of well established parasitic forms may require the maintenance, in the test-tube, of fairly constant tensions of these gases, corresponding somewhat to those in the body fluids, or even to those within the host cell. By way of an example, it may be stated that the isolation of *Tr. lewisi* from infected blood is helped remarkably by placing the inoculated tubes in an atmosphere containing from 5 to 10% CO₂. The cultures thus obtained are incomparably richer than those kept in the air. The striking results of Nowak,² McNeal,³ Fabyan⁴ and later of Wherry and Oliver⁵ on so-called partial tension isolations find their explanation, not merely in the decrease in oxygen as supposed, but also in the CO₂ supplied by the associated Hay bacillus.

It follows from what has been said that whenever an organism is cultivated in a tube or flask a considerable change in the composition of the air or gas medium is inevitable. Given an aerobic organism in a sealed tube, it is only a matter of a few hours, as a rule, for the oxygen to disappear and be replaced by a definite amount of carbonic acid. Pasteur⁶ recognized this fact as early as 1861, and especially developed it in his work in 1863. In general, it is assumed that the effect of these gaseous products is nil, and that any alteration in morphology, in spore production, or in functional activity is the direct result of changes in the culture medium. The assumption is not valid, and due consideration must be given to the changes in the over-head air and to the resulting concentration of CO₂ in the medium, not overlooking the possible presence of an injurious gas, such as acetaldehyde, or of other oxidation products, such as peroxides.

Without going into unnecessary detail, it may be stated that the study of gas changes produced by micro-organisms was long in abeyance, though initiated by Lavoisier and developed by Pasteur in his work on fermentations (1859, 1861 et seq.). It was resumed in the imperfect work of Vandevelde⁷ on the Hay bacillus. This was soon followed by the study of E. Buchner⁸ on the Fitz bacillus; of Lübbert⁹ on Staphylo-

² Ann. de l'Inst. Pasteur, 1908, 22, pp. 541-556.

³ Jour. Infect. Dis., 1910, 7, pp. 469-475.

⁴ Jour. Med. Res., 1912, 26, pp. 441-487.

⁵ The Lancet Clinic, 1916, 115, p. 306; Jour. Infect. Dis., 1916, 19, pp. 288-298.

⁶ Compt. rend. Acad. Sc., 1861, 52, pp. 1260-1264; 1863, 56, pp. 734-740.

⁷ Ztschr. f. physiol. Chem., 1884, 8, pp. 367-390.

⁸ Ibid., 1885, 9, pp. 380-415.

⁹ Biologische Spaltpilzuntersuchung. Der Staphylococcus pyogenes aureus und der Osteomyeliticoccus, Würzburg, Stahel, 1886, pp. 37-42.

coccus; of Escherich¹⁰ on *B. coli* and *B. lactis aerogenes*; and of Baginsky¹¹ on *B. lactis aerogenes*.

The work of Hesse¹² is particularly deserving of attention because he demonstrated CO₂ production and O₂ absorption by 8 organisms which were not of the aerogenic type. These results were questioned, at first by Scheurlen,¹³ who denied that bacteria could respire the same as animals, and, like Buchner, he thought that the CO₂ was produced by the action of acids on the sodium or calcium carbonate of the medium. Eventually, however, he confirmed Hesse's work by finding that every one of 141 strains of bacteria which he examined produced CO₂. From this period on, most of the work on gas exchange concerns *B. coli* and other so-called gas producers, and hence needs no special consideration at this point.

The methods which have been employed in the study of gas changes by bacteria as a rule have been somewhat complex, and it is perhaps due to this that so few workers have ventured into this field. When we began our studies, we soon realized the need of a technic which would not only permit accurate manometric observations over extended periods of time, but also would allow easy withdrawal of samples of the contained gases for the purpose of analysis. It was desirable to develop methods which would be equally available for the study of the respiration of organisms when grown on solid or liquid mediums, in the tube, or on plates in jars; either in an atmosphere of air, or in varying concentrations of O₂, CO₂, N₂, etc. The methods which we were thus led to develop, including a simple process of manometric analysis, have been utilized in the study of various organisms, bacterial and protozoal, and also of plant tissue, and the results of these investigations will be given in the several papers which will follow. This paper will therefore be restricted to the description of apparatus and the discussion of the methods employed.

CULTURAL CONDITIONS

Culture Medium.—The medium employed can be of any type desired, solid or liquid. We have used chiefly agar, either plain or with additions of glycerol, glucose, serum or defibrinated blood. Further details as to the medium used will be given in connection with the various organisms studied. The

¹⁰ Die Darmbakterien des Säuglings und ihre Beziehungen zur Physiologie der Verdauung, Stuttgart, Enke, 1886, pp. 128-133.

¹¹ Ztschr. f. physiol. Chem., 1888, 12, pp. 434-462.

¹² Ztschr. f. Hyg. u. Infektionskr., 1893, 15, pp. 17-37; 183-191; 1897, 25, pp. 477-481; Arch. f. Hyg., 1897, 28, pp. 307-311.

¹³ Arch. f. Hyg., 1896, 26, pp. 1-29; Internat. Beitr. z. innere Medicin. Zur Feier 70 jährigen Geburtstages E. v. Leyden, Berlin, Hirschwald, 1902, 2, pp. 205-207.

ordinary medium (10 c.c.) was measured out into sterile tubes which were then autoclaved at 110 degrees for 20 minutes; after which, the agar was slanted so as to have a surface of 100-110 mm. in length. With a tube 20 mm. in diameter and calculated on the basis of an ellipse, this gives on an average about 18 sq. cm. of surface; figured as a parabola, it would represent about 15 sq. cm.

The ordinary inoculation is made by transferring a loopful of a young agar or broth culture, grown in the incubator for from 16 to 18 hours. In special instances, as in the case of the tubercle bacillus, the transfer is made from a good culture by means of a spatula. Care was always taken to spread the inoculum all over the surface. Obviously, for purposes of comparison, it is desirable to inoculate approximately the same number of viable organisms. This, however, is not always realized, and the irregularity which, at times, may be seen in manometric readings, in parallel tests, finds its explanation in the varying number of organisms at work.

Culture Tubes.—An important consideration in the matter of the culture tube is the volume of the contained air. While it is possible to obtain manometric readings with small tubes, they are not suited for the withdrawal of 10 or 20 c.c. of the gas for analysis. For this and other reasons, it was found best to employ tubes of larger capacity. The 7 types of culture tubes described below were used for direct attachment to the manometer. The tubes should be thick-walled and can be made of ordinary glass, though pyrex or resistant glass may be preferable.

Type 1: The plain tube, (fig. 1 *A*) 20 x 200 mm., was used at times. When empty, the air capacity of such tubes ranged from 55-65 c.c. On an average perhaps, with 10 c.c. of agar present, the air volume was about 50 c.c. Shorter tubes, 20 x 150 mm., are necessary whenever the anaerobic bottle or jar is employed.

Type 2: The *h* tube (fig. 1 *B*). This was made by sealing into a 20 x 200 mm. tube a parallel side arm of the same diameter. The air capacity of the empty tube was thus increased to 105 to 110 c.c., or practically double that of the plain tube. The side tube enables one to introduce alkali for absorption of CO₂; or to receive water, H₂SO₄ or such other reagent as it is desired to have present. A somewhat similar tube, it may be added, was used by Bertrand¹⁴ in his study of the oxidizing action of laccase.

Type 3: The trident or 3-prong tube (fig. 1 *C*). This is made by sealing two parallel side arms to the main tube, the diameters being the same as above. The air capacity is thus increased to about 150 c.c. The 2 arms permit the use of 2 different reagents at the same time.

Type 4: Bulb tubes (fig. 2 *A*). These tubes measure 20 x 200 mm. and have bulbs of 50 or 100 c.c. capacity blown in the upper part.

Type 5: Tubes with ground caps (fig. 2 *B*) similar to the form described by Smith.¹⁵

Type 6: Side arm tubes (fig. 2 *C*). In this case, a side arm about 5.2 x 60 mm. was sealed into a 20 x 200 mm. tube. This type was found to be very useful, particularly in the study of the different methods of sealing tubes, as will be brought out in the work on the tubercle bacillus.

Type 7: Culture analyzer tube (fig. 3). This is an *h* tube (no. 2) provided with a side arm, as in No. 6, and a solid glass stopper; also with a tail-cock

¹⁴ Ann. de l'Inst. Pasteur, 1912, 26, p. 857.

¹⁵ Trans. Am. Physicians, 1898, 13, p. 417.

1. *Novy Anaerobe Bottle*:¹⁶ This should be of the large size with an internal diameter of 10 cm. and an inner height, to the shoulder, of 20 cm. A larger sized bottle measuring 12 x 25 cm. can be used to advantage. The left arm of the bottle is sealed to a one-way glass cock, which, like the manometer, terminates in a narrower tube, 5.2 x 50 mm. By means of a No. 25 rubber stopper the bottle can then be connected with a manometer. The opposite arm of the bottle is sealed to a 3-way tail-cock, the tube being bent downward so that this cock is below the shoulder of the bottle and points backward. This bottle with the attached manometer is shown in fig. 5.

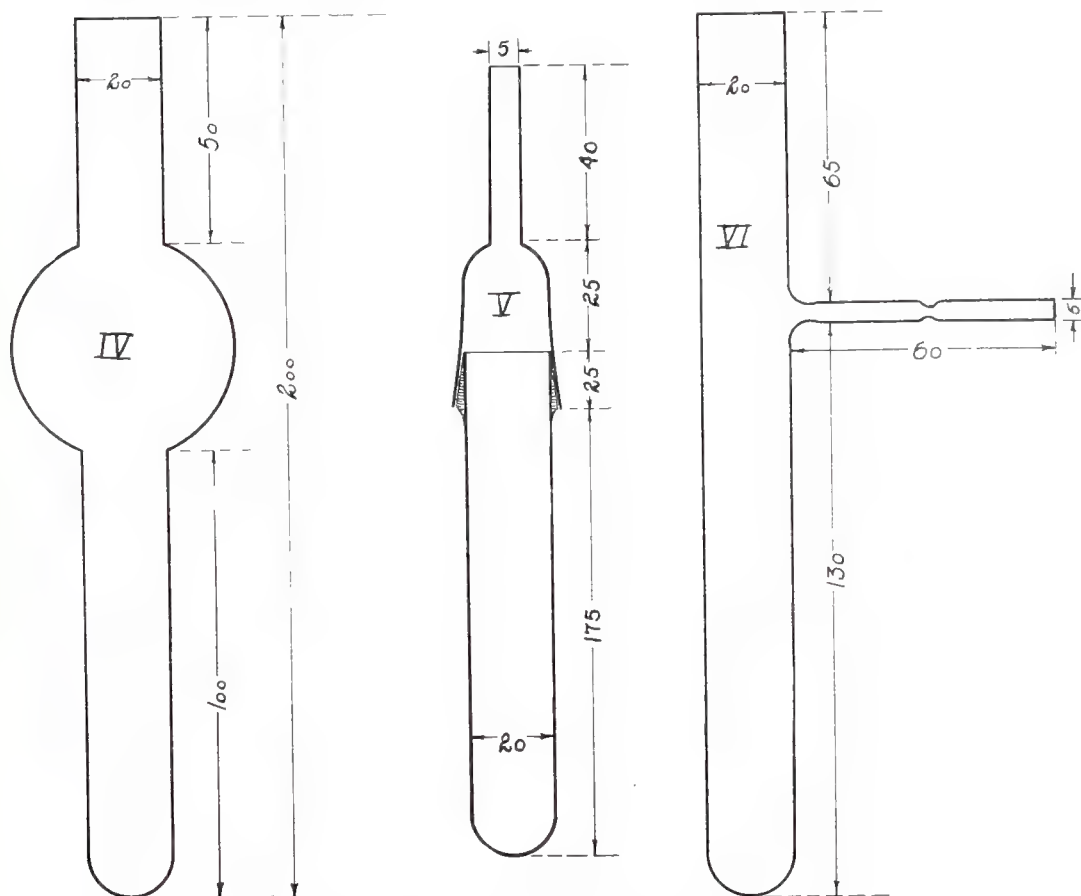


Fig. 2.—Culture tubes; A, bulb tube; B, capped tube; C, side-arm tube.

The bottle mentioned can be used advantageously without being attached to the manometer. To remove a sample of the contained air or gas for analysis, the gas buret is connected to the lower end of the tail-cock, the same as when drawing gas through cock No. 3 of the manometer.

One or more culture tubes, 20 x 150 mm., can be placed in the bottle which can then be used with air, or can be filled with any desired gas. In order to develop quickly full aqueous tension in the bottle it is necessary to place

¹⁶ Novy, F. G.: *Centralbl. f. Bakteriologie*, 1894, 16, pp. 566-571, figs. 1 and 3; *Laboratory Work in Bacteriology*, Ann Arbor, Wahr, 1899, p. 314.

on the bottom some distilled water (1 c.c.). An excess should be avoided because of the solubility of CO_2 . Finally, a good rubber band is slipped over the main stopper to hold it in place. The full capacity of the bottle should be determined in the manner described below. It usually varies from 1,200 to 1,500 c.c.

2. *Novy Anaerobe Jar*:¹⁶ Of the two types of anaerobe jars, the one with a special cock for vacuum work is the best adapted for gas studies. The tail end of the cock can be connected with the manometer by means

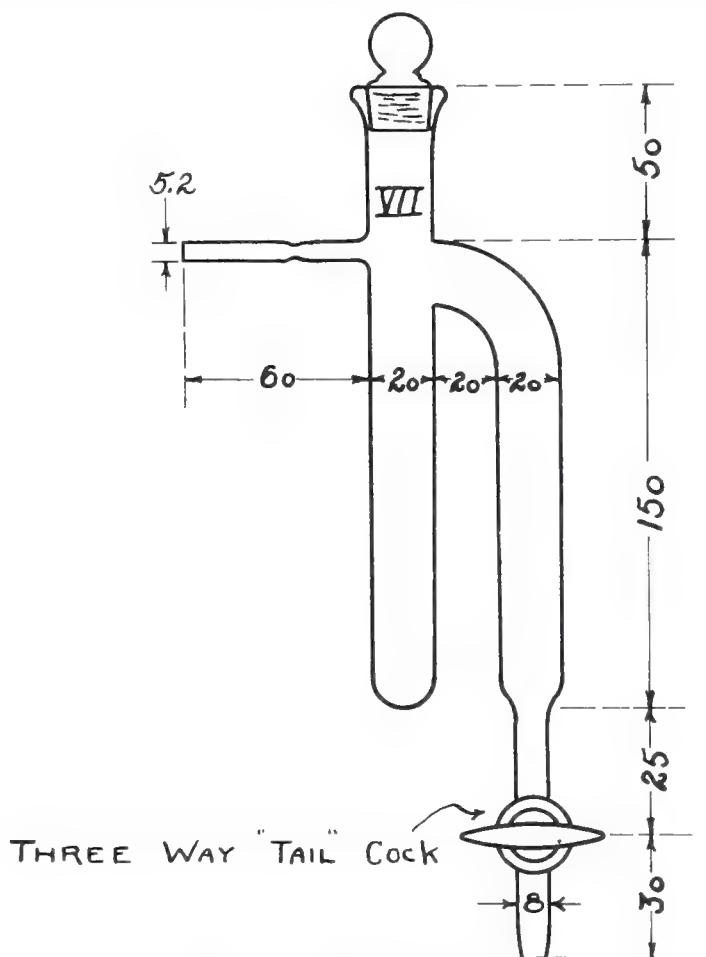


Fig. 3.—Culture analyzer tube.

of a No. 25 rubber stopper, while the head is closed with a plugged stopper (fig. 6). The jar can be used, the same as the bottle, without the manometer. In that case, the sampling of the contained gas is not as convenient, but it can be done perfectly, as will be shown later.

The lower half of the jar should have an internal diameter of 13 cm. and the internal height should be 12 cm. A taller jar can be obtained with the lower part measuring 13 x 20 cm. inside.¹⁷ The organism to be tested can

¹⁷ These jars, as well as the other glass apparatus described in this paper, can be obtained from Greiner and Friedrichs, Stützerbach in Thüringen, Germany.

be grown on the surface of plates, or in culture tubes 20 x 150 mm. About 1 c.c. of distilled water should be placed on the bottom of the jar in order to provide quickly the necessary aqueous tension. After greasing the flanges with either of the lubricants mentioned later, a rubber band (16 x 130 mm.) is applied to the circumference. Eight small vises,¹⁸ the jaws of which are covered with pieces of slit rubber tubing, are then carefully and evenly tightened to the flanges of the jar. The main stopper should be firmly wired in place, using wooden wedges, if necessary, to tighten the wire.

With very little care, the jar or bottle can be made perfectly gas tight. When filled with O_2 or CO_2 or with varying mixtures of gases and kept at 37 C. for months, no loss in the gas content can be detected.

The exact capacity of the clamped jar, including that of the cock, should be determined. For this purpose, after weighing the clamped jar empty, dis-

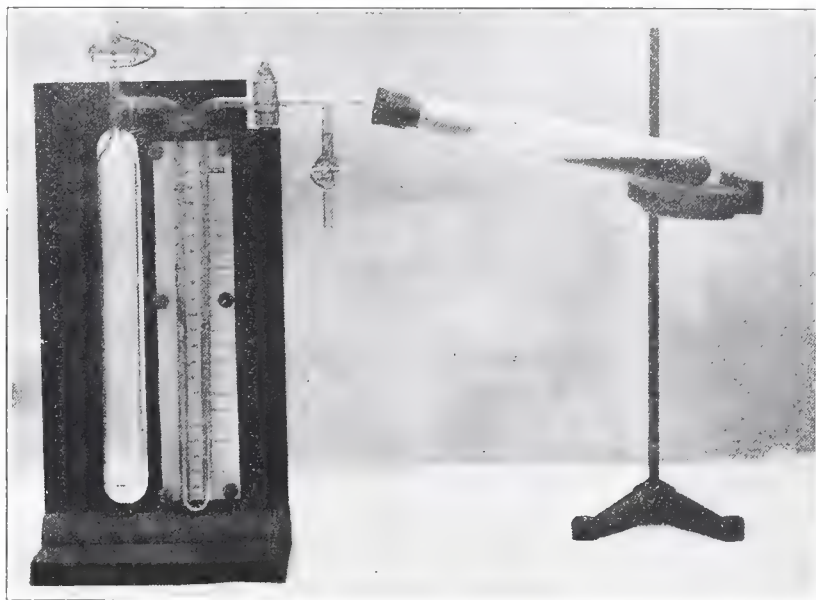


Fig. 4.—Manometer and support with *h*-tube.

tilled water is run into the jar till it is full, from tip to tip, and the weight of the water determined. This weight divided by the density of water at t degrees gives the volume capacity at 0 degree 760 mm. The jar of proper dimensions has a capacity of about 2,200 c.c., while that of the tall form is about 3,300 c.c.

The anaerobic jars after they have been in the hot room for a week or more are opened with some difficulty. It is possible to loosen the top piece by tapping, with a wooden mallet, a sharp knife which is held against the joint, but some breakage occurs. The jars can be opened easily by means of a special excentric lever which is applied to the upper flange under moderate but sustained pressure.

Rubber Stoppers: The inoculated culture tubes (types 1 to 4) were attached to the slanted tip of manometers by means of selected rubber stoppers, No. 3 or No. 4 size. A dry stopper does not give a perfect contact with the glass,

¹⁸ Phoenix vise No. 1, Phoenix Hardware Co., Buffalo, N. Y.

and, as a result, leakage is bound to occur. Thorough cleansing of the stoppers is essential. For this purpose they were first boiled in dilute alkali and rinsed with water, then boiled in dilute acid, and again repeatedly washed with water. Finally, they were placed in glycerol and autoclaved. They were then kept in glycerol.

Before use, the excess of glycerol is removed from the bore; the stopper is then attached to the manometer so as to rest firmly against the small bead on the end piece. The cotton plug on the culture tube is cut off, flamed and pushed within the tube, which is then attached to the rubber stopper. Some glycerol, if necessary, is applied to the stopper before insertion into the tube. It serves to make a firm seal, and has the advantage over petrolatum in that it is readily removed in subsequent cleansing operations.

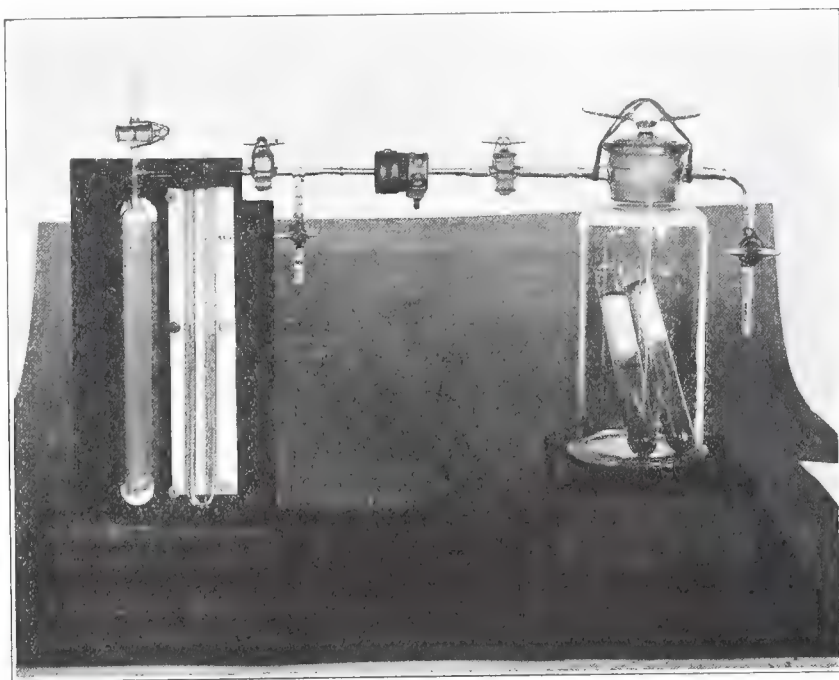


Fig. 5. --The Novy anaerobe bottle as a respiratory chamber, attached to a manometer.

CO₂ Absorption by Rubber: The use of a rubber stopper in connection with gas work is not without objection, especially when the experiment is of long duration. Hoppe-Seyler,¹⁹ as long ago as 1886, demonstrated that appreciable diffusion of gases may take place through a 3 cm. rubber stopper inserted into a 22 mm. tube, and covered externally with a mixture of fat and wax. Haldane²⁰ more recently pointed out that the use of a rubber stopper leads to a slow absorption of CO₂, but gave no details.

The permeability of thin rubber to gases has been studied by Edwards and Pickering.²¹ Their results show that taking the permeability of hydrogen as 1.0, the value for CO₂ is 2.9, for O₂ is 0.45 and for N₂ it is 0.16, while that of water vapor is about 50.

¹⁹ Ztschr. f. physiol. Chem., 1886, 10, p. 424.

²⁰ Jour. Pathol. & Bacteriol., 1920, 23, p. 449.

²¹ Publication No. 387, Scientific Papers of the Bureau of Standards, Washington, D. C., 1920.

The rapid absorption of CO_2 by rubber is easily demonstrable by means of the manometer. Thus, for one experiment, a new untreated No. 3 stopper was cut up into small pieces and placed inside of a side-arm tube; the tube, after having the mouth sealed in the blast lamp, was attached to a manometer. It was then evacuated to -720 mm. and filled with CO_2 , and placed at 38°C . Negative pressure rapidly developed: in 6 hours, the reading was -111 mm., and in 24 hours it was -223 mm. It was thus evident that the rubber, in 100% CO_2 , had a marked absorbing power. With lower concentrations the solution is not so rapid, for a similar test with 13.5% CO_2 gave a reduction, in 48 hours, of only 16 mm.

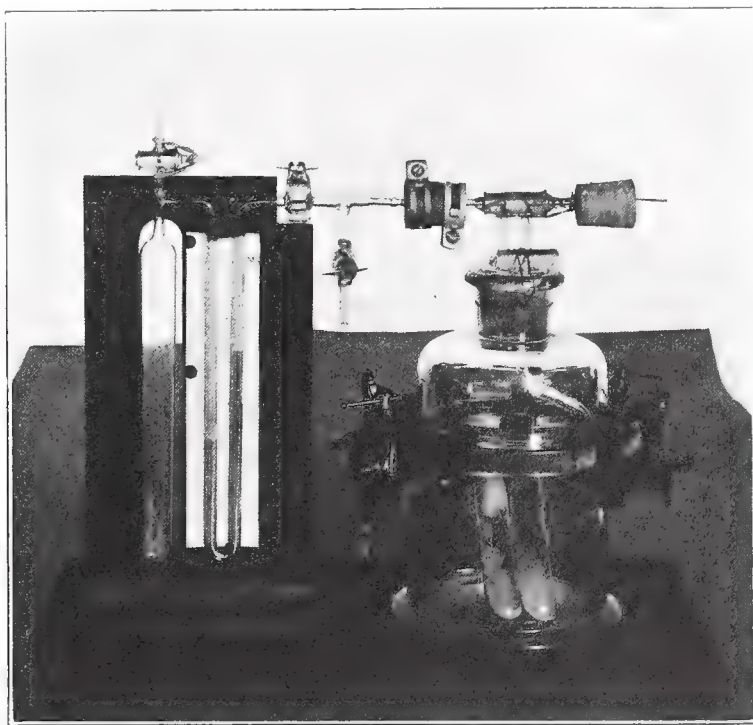


Fig. 6.—The Novy anaerobe jar as a respiratory chamber, attached to a manometer.

A simple demonstration of the diffusion of CO_2 can be obtained by passing through a rubber tube, attached to a manometer, a current of the gas under a pressure of about 50 mm. After the air has been expelled, if a clamp is applied to the rubber tubing, the pressure soon begins to fall, and then becomes negative, easily reaching -50 mm. or more.

Further evidence of the absorption of CO_2 by the rubber stopper, as actually used, will be presented in tables 9 and 10, part II. It is clear, therefore, that the rubber stopper connection as described above must be expected to influence the findings of CO_2 and also the manometric results. The extent to which this occurs will be brought out later.

Without doubt, it would be preferable to have the culture tubes provided with ground glass caps (fig. 2*b*) which can be either sealed on to the end of the manometer, thus giving an all-glass connection, or attached by means

of the No. 25 rubber stopper mentioned below. The all-glass connection, however, is by no means necessary, and equally good results may be obtained with the side-arm tubes.

It is because of these considerations that we have been led to the use of the side-arm tube, type 6. The mouth of the tube can be closed with sealing-wax, or with a ground glass stopper, or sealed in the flame. A paraffin seal is gas tight, but it cannot be used unless supported by pins which should be passed through the cotton plug before the latter is dipped in paraffin. The connection of the side-arm with the manometer can be made by sealing in the blast-lamp, but it is much more convenient to employ a No. 25 rubber stopper as described in the work on the tubercle bacillus. As will be shown, when the end of the manometer is brought into contact with the side-arm, the rubber connector presents practically no surface for absorption of CO_2 , and consequently the results are satisfactory. Ordinary rubber tubing should never be used to make this connection.

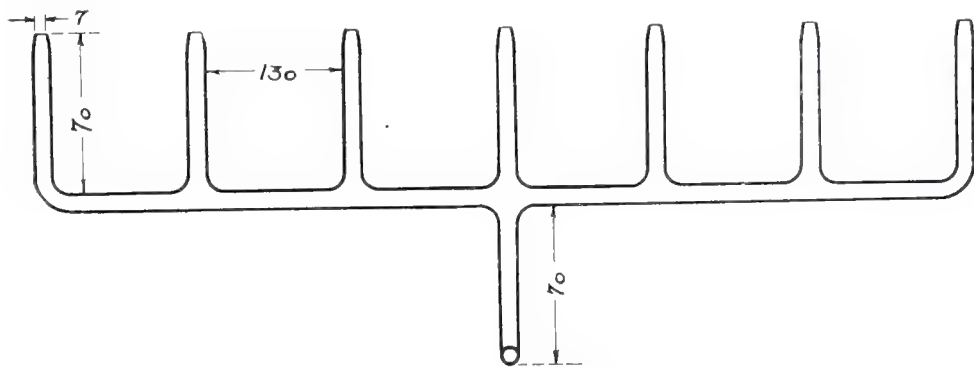


Fig. 7.—Glass rake for evacuating and refilling culture tubes.

The side-arm tubes can be attached to manometers which have the inclined tip. It is preferable, however, to attach them to the straight or horizontal tips. They should be supported on a shelf or stand so as to prevent any sag or rotation, and at the same time to remove any stress from the manometer.

REFILLING WITH AIR OR OTHER GASES

In the manipulative work incidental to the study of the gas changes in the culture tube, or in the anaerobic jar, it is necessary to be able to exhaust and to replace the gas contents with fresh air or with varying tensions of O_2 , CO_2 , N_2 or other gases.

The procedure followed, in the case of culture tubes which were attached to manometers, was to place these on the table, side by side. Frequently 6 or 7 were treated at the same time. A common connection was established by means of a 7-branch glass rake, or multiple T, shown in fig. 7. Each arm of the rake was connected to the lower end of tail-cock 3 on the corresponding manometer. Before attaching the rake to the manometers, it is advisable to wet the inside of the tubing with sterile distilled water, and to insert a loose plug of sterile cotton into each arm.

The common end or handle of the rake was joined by rubber tubing, provided with a screw clamp, *a*, to a filter tube (1.0 x 30 cm.) containing sterile cotton, and this in turn was connected to the end *A* of the special 4-prong connector (fig. 8).

The two arms, *B* and *C*, of this connector were provided with stop-cocks (Nos. 4 and 5). The arm *B* was connected with the N₂ purifying train by a rubber tubing bearing a screw-clamp, *b*, while the other arm was similarly joined to the CO₂, or O₂ supply, and controlled by the screw-clamp, *c*. In ordinary refilling, the air was admitted through *c*.

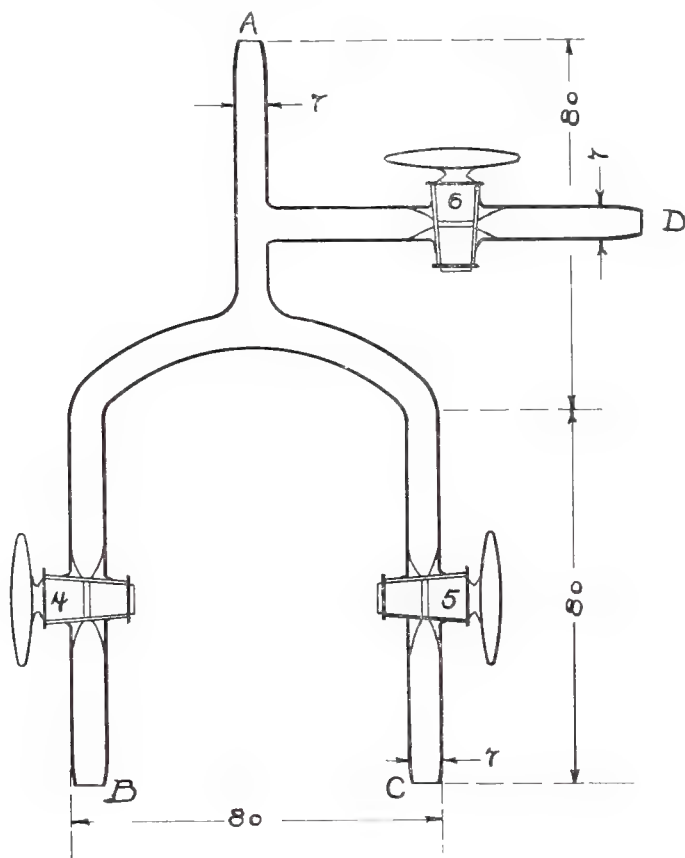


Fig. 8.—Connector which connects at *A* with the rake; at *B* with nitrogen; at *C* with oxygen or CO₂ supply; at *D* with the suction apparatus.

The side-arm, *D*, carried a stop-cock (No. 6), and was connected with the evacuating apparatus which consisted of a Chapman water pump, a vertical manometer, and a Woulff bottle. One tubulure of the latter was closed with a rubber stopper through which was inserted one of the main arms of a 3-way T cock (No. 7). The midarm of this cock, horizontal in position was joined to the side-arm, *D*, of the connector. The upper main arm was attached to the descending limb of a vertical manometer (800 mm.). Another tubulure was connected with the pump, the action being controlled by means of a 3-way tail-cock (no. 8).

Instead of the special connector mentioned above, an ordinary **T** may be used, the midarm being connected to the Woulff bottle, and the distal end having a short piece of rubber tubing which is closed with a screw-clamp (*c*).

The method of evacuating and refilling the culture tubes with air is as follows:

1. Open cocks 2 and 3 on the manometers; 7 and 8 on the suction apparatus, and close clamps *a*, *b* and *c*. Then start the pump and evacuate to -700 mm.

2. When this evacuation is reached, open carefully and slightly clamp *a*. The air is thus drawn out of the culture tubes and the Hg in the compensation manometers rises. As soon as it reaches about -200 mm., the clamp *a* is closed to prevent the Hg from being sucked out of the manometers. Then close all of the No. 2 cocks, reopen clamp *a*, and complete the evacuation of the tubes to -700 mm.

3. Now give cock 7 a $\frac{1}{4}$ turn backward. This shuts off further pump action but leaves the vertical manometer in communication with the culture tubes. Then slightly open clamp *c*, thus admitting air into the pipe line and tubes. And, as a result, the Hg in the vertical manometer drops.

4. When the Hg falls to zero, close clamps *a* and *c*, and then open cocks 2. The Hg in the compensation manometers now drops to zero. When this is accomplished, close the No. 2 cocks.

5. Then by a $\frac{1}{4}$ forward turn, bring back cock 7 to its original position. A partial vacuum is at once reestablished in the pipe line and in the culture tubes, and the evacuation is continued until -700 mm. is reached.

6. Proceed as in 2.

7. Proceed as in 3.

8. Proceed as in 4.

Repeat steps 5 to 8, 3 more times, thus giving 5 evacuations and refills. After the last refill, close the No. 3 cocks on the manometers, and disconnect the manometers from the rake. Open the No. 2 cocks and place the manometers in the hot-room. After allowing 2 hours for the temperature to equalize, open cocks 1 and 3, equilibrate the manometers, and then close the cocks.

In some cases, as when working with tubes sealed with paraffin plugs, though these are supported by pins, it is not advisable to evacuate to more than -300 mm.; when closed with sealing-wax, it is well not to exceed -500 mm.; while with rubber stopper or glass seals, the evacuation may be pushed to -700 mm. In order to avoid the possibility of breakage, and the setting of the main stopper, when refilling anaerobe jars or bottles, it is advisable to evacuate to -500 mm. or less.

The operation of evacuation and refilling was repeated at least 10 times when working at -300 or -500 mm. and about 5 times when the highest vacuum was employed. One of the tubes was always analyzed as a control on the process. In the case of the jars, a sample of the air was withdrawn and likewise analyzed. It may be added that the complete removal of the CO_2 which is dissolved in the medium is rather difficult, and for that matter not necessary. Hence it is that the control analysis just mentioned will usually show some CO_2 due to diffusion from the medium. It may be stated further that not a single contamination of the culture in the tubes has occurred under these conditions.

The same operation was carried out when the tubes were to be refilled with pure N_2 , O_2 or CO_2 . The N_2 supply was connected with clamp *b*, while the O_2 or CO_2 source was joined to clamp *c*.

If it is desired to introduce into a tube a certain percentage of CO_2 , for example 40%, this can be readily done. Assuming the barometer to read 750, and the thermometer 22.3 degrees, the corresponding aqueous tension is 20 mm. of Hg. Hence $B - T = 750 - 20 = 730$ mm. and 40% of this = 292. This value represents the partial tension of the gas to be admitted, provided it is dry. The washed gas, however, is more or less saturated with moisture, and hence 292 mm. would actually represent less than 40% of the desired gas. It is therefore preferable to take 40% of the observed barometric pressure, or —300.

The filling of the tubes is carried out very much as in steps 1 —4. The cocks and clamps are attended to as in step 1. The pump is started, and when the Hg in the compensation manometers reaches about —200 mm., cock 7 is given the $\frac{1}{4}$ turn backward. Then clamp *a* is shut off and the No. 2 cocks are closed, after which clamp *a* is again opened, and cock 7 is brought back to its original position. As soon as the vertical manometer reaches —300 mm., it is again shut off by the $\frac{1}{4}$ turn, and clamp *a* is closed.

The culture tubes now have the desired negative pressure, but before admitting CO_2 the pipe-line must be fully evacuated. Otherwise the CO_2 would drive the air, which remains in the line, into the culture tubes, and consequently a considerable lower percentage of CO_2 than was intended would be obtained.

This is avoided by opening cock 7 and evacuating the line as far as possible. This cock is then closed by the $\frac{1}{4}$ turn backward. Clamp *c*, to which the CO_2 supply is attached, is then slightly opened to admit the gas into the line. When the Hg of the vertical manometer drops to zero, the clamp is closed and the line is evacuated and refilled as before. This operation is repeated once or twice more to insure that the line is filled with pure CO_2 .

Finally, when at the last filling the Hg of the vertical manometer has dropped to zero, clamp *c* is closed and *a* is opened slightly and the CO_2 is admitted into the culture tubes. Clamp *a* is then closed and the No. 2 cocks are opened, with the result that the Hg in the compensation manometers falls but does not always reach zero. Hence, additional CO_2 is admitted by slightly opening clamp *a*, and, if necessary, clamp *c*, until zero pressure is reached. The clamps are then closed, likewise cocks 3, and the manometers are disconnected. The culture tubes now contain the desired 300 mm. of CO_2 . In order to make certain of the exact amount present, one of the tubes is analyzed. In the case of jars, a sample of the contained gas is withdrawn and likewise analyzed.

If, on the other hand, it is desired to have 40% of O_2 in the tube, this may be done by alternate evacuation and filling with moist O_2 , following the procedure for refilling with air, but instead admitting O_2 after each evacuation. When all of the air has been displaced, the apparatus is evacuated to about —450 mm., and the pump is disconnected by a $\frac{1}{4}$ turn backward of cock 7. Clamp *a* is then closed, and the line is now fully evacuated and filled with pure N_2 , in the same manner as given above under CO_2 . Finally, the pure N_2 is admitted into the culture tubes or jar until the manometer shows zero pressure. The mixtures thus obtained are fairly correct, but the exact composition must be determined by analysis.

By means of the foregoing procedure, it is possible to introduce into the culture tube or jar any desired amount of a given gas. Thus, in the work with *B. tuberculosis*, the oxygen content was varied from 0.5 to 100%. Similarly, the effect of CO_2 in concentrations of 10, 20, 40, 60, 80 and 90% could be studied.

Nitrogen.—Commercial tanks of nitrogen, made by the air reduction process, were employed in this work. Such nitrogen is never pure but contains about 6% of O_2 and at times a trace of CO_2 . It is necessary therefore to remove completely these gases by passage through alkaline pyrogallate. For this purpose, 10 absorption towers (40 cm. high) were set up in series, and filled with alternating thick layers (25 mm.) of glass beads and thin layers (5 mm.) of fine garnets. The latter served as baffling plates, and materially aided in the otherwise slow and incomplete absorption of the oxygen. Through this system, filled with alkaline pyrogallate, the nitrogen could be passed at the rate of 200-300 c.c. per minute, with complete removal of its oxygen contents. The efficiency of the washing was controlled by analysis of samples collected in a Bailey bottle.

Oxygen.—This was obtained in the small tanks as used for medical purposes. On analysis it was usually found to contain no CO_2 and 97-98% O_2 . To remove any trace of CO_2 which might be present, the gas was always passed through a Drechsel wash bottle containing 10% KOH.

Carbon Dioxide.—This was usually made in a Kipp generator out of marble and dilute HCl. The gas was washed by passing through 3 Drechsel bottles containing saturated solution of Na_2CO_3 . The efficiency of the washing process was controlled by testing the gas with $AgNO_3$. For some purposes, the commercial CO_2 in tanks was employed. Analysis showed it to be practically pure, the residual gas after absorption of the CO_2 being too small for transfer into pyrogallate.

Compressed Air.—This was taken from the laboratory mains and usually was purified by passing through KOH and then through H_2SO_4 , which reagents were contained in Friedrich's spiral wash bottles. In special cases, the compressed air was sent through a long tube filled with soda lime, and then saturated with aqueous vapor as described in connection with the aeration experiments on *B. tuberculosis*.

THE COMPENSATION MANOMETER

In the study of gases, it is essential to be able to observe the pressure changes which take place within the culture tube or jar, for only in this way is it possible to follow the reaction which takes place, hour by hour, or day by day. The manometer, usually, not only reveals whether an organism is alive and growing, but also indicates the point when growth or respiration ceases.

Lübbert,²² in his study of the gas exchange of the staphylococcus, used an open Hg manometer which was attached to the culture flask. He gave no readings, and merely stated that in one experiment a marked decrease in volume was shown by the manometer. Godlewski,²³ a decade later, likewise used a Hg manometer in connection with cultures made in vaccum, as did, still later, Wolf and Harris²⁴ and Bushnell.²⁵

²² Footnote 9, p. 37.

²³ Abderhalden's Handb. d. biochem. Arbeitsmethoden, 1910, 3 (2), p. 519.

²⁴ Jour. Path. & Bacteriol., 1917, 21, pp. 386-452.

²⁵ Jour. Bacteriol., 1922, 7, p. 384.

Kostytschew²⁶ made temporary use of a manometer when drawing samples of gas for analysis. Recently, Rockwell and McKhan²⁷ made limited use of an Hg manometer attached to a culture of gonococcus. Undoubtedly, many others have made use of this instrument, but such records are not easily found.

The so-called manometric principle has been used in the estimation of sugar in urine, in the form of a variety of fermentation tubes, as for example those of Fleischer,²⁸ Einhorn²⁹ Lohnstein,³⁰ Wagner,³¹ Goldmann³² and Söhle.³³ At best, these can be characterized as crude attempts at measuring the volume of the gas evolved, or of the pressure produced.

Recently methods for the automatic registration of the gas pressure produced by organisms have been devised for bacteria, by Fleming and Clemenger;³⁴ and, for yeast, by Sieburg³⁵ but their applicability is limited practically to the anaerobic aerogenic forms, and their value is questionable. By substituting vaseline for the paraffin plug used by the former, Brown³⁶ was able to devise a syringe method of micro-gas analysis.

The real manometric principle was utilized by Barcroft and Haldane³⁷ and by Barcroft³⁸ for the estimation of O₂ and CO₂ in small quantities of blood. Their instrument, modified by Brodie,³⁹ has been used for diverse respiration studies, notably those of Warburg,⁴⁰ of Rona⁴¹ and others.

The short U-shaped manometer naturally suggests itself as the form best suited for experiments under ordinary pressure conditions, that is, with pressures not exceeding 250 mm. As commonly used, the free end is open, and hence the reading is influenced by the continual variation in the normal barometric pressure. Obviously, for comparative readings made at short intervals and perhaps extending over a number of days

²⁶ Centralbl. f. Bakteriöl., II, 1904, 13, pp. 490-503; 577-589.

²⁷ Jour. Infect. Dis., 1921, 28, p. 255.

²⁸ München. med. Wchnschr., 1887, 34, p. 601.

²⁹ Virchow's Arch. f. path. Anat., 1885, 102, pp. 263-285; Deutsche med. Wchnschr., 1888, 14, p. 620; 1891, 17, p. 463; New York Med. Rec., 1887, 31, pp. 91-94.

³⁰ München. med. Wchnschr., 1899, 46, pp. 1671-1675; Berl. klin. Wchnschr., 1898, 35, pp. 866-868; Allgem. med. Zeitung, 1898, 1899, 1900.

³¹ München. med. Wchnschr., 1905, 52 (2), pp. 2327-2329.

³² Ber. deutsch. Pharm. Gesellsch., 1906, 16, pp. 110-115; 1907, 17, pp. 62-66.

³³ Chem. Zeitung, 1911, 35, p. 871.

³⁴ Brit. Jour. Exper. Path., 1920, 1, pp. 66-69.

³⁵ Biochem. Ztschr., 1922, 130, pp. 459-462.

³⁶ Jour. Exper. Med., 1922, 35, pp. 667-684.

³⁷ Jour. Physiol., 1902, 28, pp. 232-240.

³⁸ Ergeb. der Physiol., 1908, 7, pp. 772-775.

³⁹ Jour. Physiol., 1909-10, 39, pp. 391-396.

⁴⁰ Biochem. Ztschr., 1919, 100, pp. 230-270; 1923, 1924.

⁴¹ Ibid., 1922, 128, p. 174; 134, pp. 146-162.

or weeks, a constant barometric pressure is essential. This can be obtained by sealing the open end, but in so doing the sensitiveness of the manometer is greatly impaired. Hence, the readings of a closed-end manometer cannot be expected to correspond exactly with those of an open one. Indeed, the readings of these two types may vary considerably; a fact which can be readily ascertained by attaching the two manometers, side by side, to a common aspirator. In such comparison a closed-end, capillary manometer may be found to show only $\frac{1}{2}$ or $\frac{1}{3}$ of the actual pressure.

The sensitiveness of the closed-end manometer can be increased, or compensated, in two ways: (1) by increasing the volume of the air confined in the sealed arm, and (2) by decreasing the internal diameter of the manometric tube. Or, stating the matter more precisely, the sensitiveness of the closed-end manometer varies directly as the volume of the air in the sealed end, and inversely as the square of the radius of the manometric tube (table 1). While, therefore, the readings of a closed-end manometer cannot be made to agree exactly with those of one with an open end, they can be brought, however, by suitable compensation, to so close an agreement with the latter as to be practically correct. Furthermore, when desirable, the readings can be corrected by means of the correction factor, which will be taken up presently.

Description of Manometer.—The construction of this instrument will be readily understood from fig. 9. The compensating air cylinder is about 25 x 240 mm., and has a capacity of about 100 c.c. It is connected above with the one-way stop-cock, No. 1, below which is inserted the U-shaped capillary manometer tube, the internal diameter of which should be about 2 mm. The arms of the U are about 250 mm. in length, and hence it is possible to work with positive or negative pressures nearly up to this limit.

The distal end of the U is connected with the one-way stop-cock, No. 2, and beyond this is a **T** the stem of which extends downward and is provided with a 3-way tail cock, No. 3, which points backward. The **T** terminates in a short glass tube which is about 50 mm. long and has an external diameter of about 5.2 mm. so as to fit perfectly the 4-5 mm. bore of a rubber stopper. This terminal tube has a small bulb, about 7.5 mm. in diameter, and about 30 mm. from the end, which fixes the position of the rubber stopper. This tube is bent slightly downward, so that when a culture tube is attached, as, for example, an agar slant, the water of condensation remains on the bottom of the tube. For use with

side-arm tubes (fig. 2 C), or with the Novy bottle or jar, this end should be horizontal, though this is by no means necessary.

The glass manometer, which is about 325 mm. high and 200 mm. wide, is mounted on a suitable board support, as shown in figs. 4, 5 and 6. A strip of millimeter ruled, cloth lined paper, properly numbered, is fastened back of the U tube, and serves as the scale.

Stop-cock 1 is used, together with No. 3, to equilibrate the Hg levels at the start of an experiment; after which it is closed, and remains

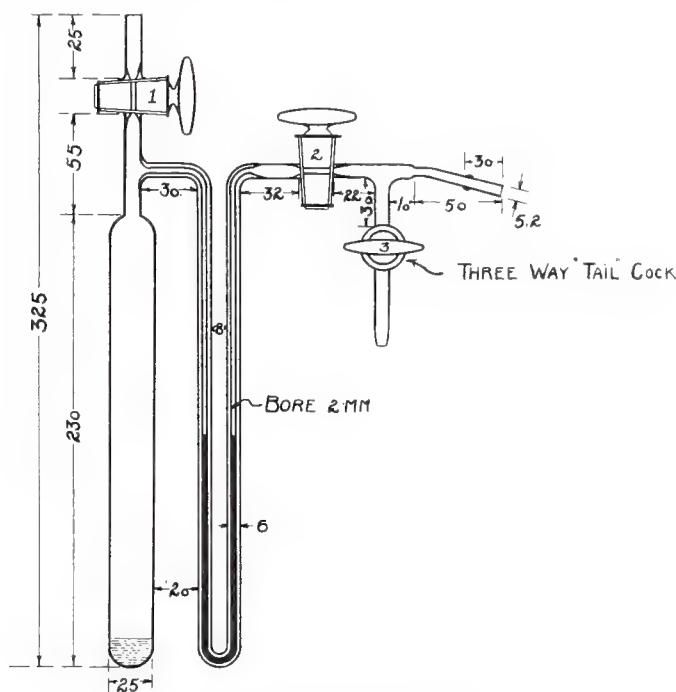


Fig. 9.—Glass parts of the manometer.

so till the end. The air in the cylinder therefore remains under the original barometric pressure at the time of equilibration.

The purpose of stop-cock 2 is two-fold: (1) to shut off the manometer when the gas pressure is likely to exceed the capacity of the U tube; and (2) to enable one to withdraw gas from the culture tube without sucking over the Hg column. Likewise, when evacuating the culture tube with the object of refilling it with fresh air, or with O_2 , CO_2 , etc., this cock must be closed. At all other times, it is kept open.

Tail-cock 3, as stated above, is used to equilibrate the Hg levels at the beginning of a test, after which it remains closed. By means of this

cock a sample of the gas content in the culture tube is easily withdrawn for analysis. It is also the means by which the gas within the culture tube is evacuated and replaced with fresh air, or with any desired gas.

Calibration of Manometer.—Before proceeding with the calibration, it is necessary to clean and dry thoroughly the inner walls of the instrument, and especially to remove any grease in or about the stoppers. It should be pointed out that for ordinary work it is by no means necessary to calibrate the manometer, since the observed readings are but 1 to 3% below the corrected values. When, however, the utmost accuracy is desired, it becomes necessary to correct the observed reading, and this is done by means of the correction factor. In the manometer, as described, it is the radius of the capillary which chiefly determines the value of this factor.

(1). Determination of the Radius of the Capillary: Some Hg is drawn into the right arm of the manometer so as to have a column approximating 20 cm. in height. The exact height of the column is then read from the scale. In case the latter is inaccurate the proper correction value for the scale is applied. The corrected height in cm. gives the value of h . The Hg is then emptied into a small beaker and weighed ($=W$ in grams).

With d as the density of Hg at t degrees; and r , the radius in cm., we have

$$W = \pi r^2 h d$$

$$r^2 = \frac{W}{\pi h d}, \text{ and } r = \sqrt{\frac{W}{\pi h d}}$$

and hence

The value of r thus obtained multiplied by 10 gives the radius in mm.

(2). Determination of Air Space in Right Arm of Manometer: At some point on the right arm of the U Capillary, for example at the 100 mm. mark on the scale, a scratch is made on the glass. Then Hg is drawn up carefully to this mark so as to fill the entire space, from the mark to the extreme tip of the arm, including the space above the No. 3 cock. The Hg is then emptied into a beaker and weighed, the temperature being noted. The volume in c.c. is computed from

$$V = \frac{W}{d \text{ at } t^\circ}$$

This volume represents the air space from the mark on the right arm of the capillary to the extreme tip. It must be taken into account when calculating the total volume of the air or gas present in the culture tube or chamber. This value in our manometers ranges from 2.28 to 3.81 c.c.

A slight additional correction may be needed to represent the volume of gas between the level of the Hg, in a given experiment, and the above mark on the capillary. This is deduced from

$$V = \pi r^2 h$$

(3). Determination of Air Volume in Compensator: By careful suction, water is drawn in until the space in the left arm of the manometer is filled, from cock 1 downward to the level of the 100 mark on the scale. This water is then measured or weighed, thus giving the volume of air in the compensating

chamber, that is, over the Hg in the manometer. This volume varies in our instruments from 93 to 114, but usually is close to 100 c.c.

(4). Correction Factor: The following calculations which we owe to Mr. M. S. Marshall will serve to illustrate the development of the theoretical true correction.

Let B = barometric reading at start (mm.)

p = unknown pressure \pm (mm.).

r = radius of capillary (mm.).

V = volume from cock 1 to 0, the left Hg level (mm.³).

v = volume between 0 and new Hg level in capillary (mm.³).

$C+$ = correction factor for positive pressure (mm.).

$C-$ = correction factor for negative pressure (mm.).

Then from the gas law $p' v' = p v$

$$p' = \frac{p v}{v'}$$

$$\text{and substituting } p' = \frac{B V}{V \pm v}$$

where $p' > B$ if $p = +$
 $p' < B$ if $p = -$

$$\text{Hence } C+ = \frac{B v}{V - v} - B, \text{ and } C- = B - \frac{B v}{V + v}$$

$$\text{Solving: } C+ = \frac{B v}{V - v} \quad \text{and } C- = \frac{B v}{V + v}$$

and since $v = \pi r^2 p/2$

$$C+ = \frac{B \pi r^2 p}{2V - \pi r^2 p} \quad \text{and } C- = \frac{B \pi r^2 p}{2V + \pi r^2 p}$$

These corrections can be calculated knowing the values of B, r, p, V . However, V need not be considered as a variable since with values of 90 and 100 c.c. the difference is but a small fraction of a mm. Likewise B need not be regarded as a variable for variations of the barometer from 730 to 750, with 740 as the main being used in the calculation, are of negligible effect.

Hence it is justifiable to leave $\pi r^2 p$ out of the denominator; and by making $V = 100 \text{ c.c.} = 100,000 \text{ mm.}^3$; and using $B = 740 \text{ mm.}$ the correction becomes

$$(I) \quad C = \frac{740 \times \pi r^2 p}{200,000}$$

$$\text{and if } p = 1 \text{ mm., } C = \frac{740 \times \pi r^2}{200,000}$$

and hence, (II) $C = 0.01162 r^2$, which is the correction factor per mm. pressure in the manometer.

Formula I can be written as $\frac{\pi r^2 p}{200,000} \times B$, and if $p = 1 \text{ mm.}$

$$\text{it becomes } \frac{3.14}{2,000} \times \frac{r^2}{\text{Vol. c.c.}} \times B$$

or, (III) $\frac{0.001571 r^2}{\text{Vol. c.c.}} \times B$ which would be the correction factor, per mm. Hg, for any barometric pressure, and any volume of air in the compensator.

Taking formula III, table 1 has been constructed. It is of interest since it shows clearly the effect of the size of the capillary and the volume of the closed end upon the delicacy of a manometer. It will be seen that, with the

barometer at 740, a manometer with a 100 c.c. compensator, and a capillary radius of 1 mm. has a correction factor of .0116 or 1.16% of the observed manometric reading. The correction factors of our manometers range from 0.0099 to 0.0282. The correction for an observed reading of 100 mm. would be in the former case 0.99 mm. and for the latter 2.82 mm.

The calibrations having been made, the manometer can then be prepared for actual use. About 1 c.c. of distilled water should be drawn into the compensating cylinder in order to have always the full aqueous tension in this confined space.

A current of dry air should then be blown or drawn through the apparatus until all of the moisture is removed from the U and the right arm. After which, Hg is drawn in so that it stands in both arms on or near the 100 line of the scale. Distilled Hg should be used in the manometer and also in the gas buret and gas sampler.

The glass stoppers are now carefully lubricated with a suitable grease. A good lubricant, which we have employed for many years, is made by dissolving 1 part of clear bees-wax in 3 parts of olive oil. When the manometers,

TABLE 1
CORRECTION FACTORS, PER MM. HG PRESSURE OBSERVED, FOR CLOSED-END MANOMETERS

$$= \frac{0.001571 \, r^2}{\text{Vol. c.c.}} \times 740 = \frac{1.16254 \, r^2}{\text{Vol. c.c.}}$$

Air Volume in Closed End, C c.	Radius of Capillary in Mm.			
	0.5	1	1.5	2
1.....	0.290	1.162	2.614	4.647
10.....	0.029	0.116	0.26	0.46
50.....	0.0058	0.023	0.052	0.0929
90.....	0.0032	0.0129	0.029	0.0516
100.....	0.0029	0.0116	0.026	0.0456
110.....	0.0026	0.0106	0.024	0.0424

anaerobic jars, etc. are to be used at about 37 C., it is advisable to use a firmer mixture (1:1). But at the higher temperature, this grease, owing to its appreciable tension, tends to spread over the adjoining glass surface. It may cover the inside of the compensator, and may eventually enter the capillary. When this condition supervenes, the manometer should be emptied, thoroughly cleaned and refilled.

An excellent grease, known as "Lubrisal," which has less tension than the one mentioned, is made of rubber and paraffin.

The stop-cocks must be held firmly in place by strong rubber bands. Otherwise, if this precaution is not taken, a leak is likely to occur because of the tendency of the stopper to lift itself. This tendency can be reduced considerably by using as little of the lubricant as possible.

Scale Correction.—The accuracy of the scale should be tested with good vernier calipers. The mm. ruled paper, as taken from the roll, was found to be correct when measured lengthwise. Measured transversely, however, 99.6 mm. of the calipers covered 100 divisions on the paper. Hence, the observed readings on a scale of this kind should be corrected by subtracting 0.4%. With low manometric values, this correction can be neglected.

Equilibration of Manometer.—The manometers with the attached tubes are placed in the hot-room at the desired temperature, for at least 2 hours before equilibrating. A constant temperature is necessary for good readings on a manometer. During this time, cocks 1 and 3 on the manometer should be kept closed. As a result, positive pressure will develop due to the expansion of the confined air or gas and to increase of aqueous tension up to the saturation point.

When a jar is to be used, with a manometer, it is advisable to place it in the hot-room over night. The next day the tubes are inoculated and placed within the jar, which is then closed and attached to the manometer. Cocks 1 and 3 are kept closed until the full aqueous tension has developed.

When the temperature and tension have been equalized, the manometer is equilibrated by opening cocks 1 and 3. But when the tubes or jars are filled with gas other than air, in order to prevent loss, the equilibration is made with the tip of stop-cock 3 placed below the level of water in a small beaker. The Hg is then oscillated a few times by applying a rubber bulb to the tip of cock 1. The Hg in the two arms of the manometer should be on the same level. This being the case, cocks 1 and 3 are then closed and rubber bands are adjusted over the 3 cocks to hold them firmly in place. The reading of a Hg barometer is taken at this time; likewise the temperature at the level of the manometer is recorded.

It is always advisable to take several readings of the manometer, at hourly intervals, in order to make certain that true equilibration has been effected. An important condition is the development of full aqueous tension, as will be shown at the end of this paper.

Observed Pressures.—In the case of rapidly growing organisms, such as *B. subtilis*, *B. coli*, etc., it is advisable to make hourly readings. When the organism is a slow grower, as for example *B. tuberculosis*, it is sufficient to take the readings at 12 or even 24 hour intervals. The reading of the Hg should be taken after a gentle oscillation of the column, which can be done easily by giving the manometer a sharp jerk while holding it and the tube in a slightly inclined position. The aerobic organisms, when grown on mediums which do not contain glucose, produce a negative pressure which rapidly rises to a maximum and then remains constant. When the organism grows fast, this point may be reached within 24 to 48 hours. It indicates that the oxygen has been reduced to a small fraction of a percent, if not actually to zero. Aerobic growth, in its simplest terms, consists in the rapid absorption of oxygen and the formation of CO_2 . The progress of this exchange can be followed by drawing a sample of the gas and submitting it to analysis.

If the negative pressure, after reaching its maximum, shows a slow steady fall, it indicates that CO_2 is being slowly produced either as the result of anaerobic respiration or of carboxylase action.

Control Tubes.—Before taking up the analytic methods, it may be well to call attention to the behavior of control tubes. These contain the same medium but are not inoculated. They are attached to manometers and are equilibrated at the same time as the inoculated tubes, after which they are kept at the same temperature as the latter.

It might be assumed that an uninoculated tube should show no manometric change, the Hg remaining at the zero level. This condition is usually realized for the first few days, but, if the incubation is continued, eventually a slowly increasing negative pressure will be observed. Examples of this will be seen in the tables in parts II and III. Analysis of such tubes reveals a decrease

in O_2 and an increase in CO_2 . Even a clean, empty tube when attached to a manometer with a glyceroled rubber stopper and kept at 37 C. for 31 days gave a —14 mm. pressure, and analysis showed a decrease in oxygen and an increase in CO_2 . In this case, the glycerol and the rubber stopper were being acted on. All this merely illustrates the general law that organic matter in the presence of O_2 is slowly oxidized. Pasteur,⁶ in 1863, showed that this was true for sterile infusions, urine, milk, blood, etc., kept in sealed flasks. He pointed out that this slow combustion of dead matter by the oxygen of the air was real though scarcely appreciable, whereas if germs were present, the oxygen was completely absorbed in a few days.

MANOMETRIC ANALYSIS

The culture analyzer (fig. 3) when connected with a manometer, as shown in fig. 10, can be used to determine the percentage of CO_2 and O_2 , and hence the respiratory quotient, in an aerobic culture. When the apparatus is filled with N_2 or H_2 , it can similarly be used to determine the $\frac{CO_2}{H_2}$ ratio or hydrogen quotient. The analyses should be made at a constant temperature, preferably in the hot-room.

Air Analysis.—This in itself is good practice in the manipulations, and at the same time demonstrates the absence of any leakage. Before use, the analyzer is first cleansed, especial care being taken to remove the grease from the stoppers. A pledget of cotton is inserted into the narrow side-arm, and the stoppers are loosely put in place, a strip of paper separating each from its seat. They are then sterilized in the dry heat oven at 200 C. The stoppers are then smeared with a 1:1 olive oil wax mixture. This is especially needed for the tail-cock which, after alkali is once used, is turned with difficulty when a thinner lubricant is employed. A few drops of sterile distilled water are placed in the main tube to supply the full aqueous tension, and at the same time the bore of the tail-cock is filled with water. The analyzer is then placed on its support and attached to a manometer by means of a No. 25 rubber stopper and hose clamps. The combined apparatus is placed on a platform which permits either rocking or fixation at any desired angle and is then transferred to the hot-room. The water in the tube is gently heated several times, with cocks 3 and 4 closed, to saturate the air with aqueous vapor, and after an hour or two, the manometer is equilibrated in the usual way.

The absorbing reagents are the same as used in the buret analysis. The pyrogallate solution is covered with a layer of paraffin oil.

Each reagent is contained in a glass cup or cylinder to which is attached a rubber tube (90 cm.) terminating in a glass stop-cock. When the reagent is to be used, the end of this stop-cock is connected by narrow rubber tubing with the tip below the tail-cock of the analyzer. A pinch-cock on the rubber tubing is very useful. On opening the stop-cock, the reagent passes through the tubing and out of the tail-cock, where it is caught in a small beaker. The air having been expelled from the connections, the tail-cock is then opened and the reagent introduced into the side-arm of the analyzer. If the manometric pressure is negative, the reagent will promptly rise into the side-arm. When there is no pressure, the reagent must be forced into the analyzer by raising the reagent cup, or by compressing an air bulb which is

applied to the cup. When there is a high positive pressure (100-200 mm.) in the apparatus, this can best be overcome by a sufficient head of Hg, which is applied to the reagent cylinder in the manner shown in fig. 10.

Since a difference of 1 mm. Hg pressure in the manometer represents about 0.14%, it would be futile to attempt the determination of the CO_2 present in atmospheric air. Hence to test the apparatus, about 7 c.c. of the pyrogallate solution are forced into the side-arm, after which the pinch-cock is applied, and the tail-cock is closed.

The apparatus is then rocked freely at times, and fixed in an inclined position so as to get the maximal absorbing surface. Manometric readings, taken at 20 minute intervals, will indicate the point when absorption is com-

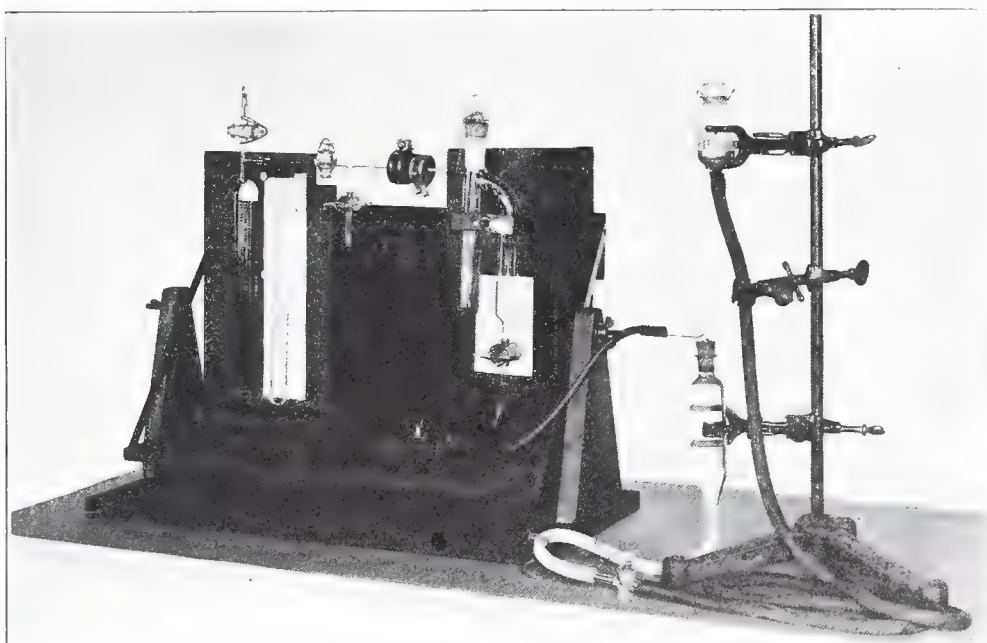


Fig. 10.—Manometric gas analysis. The analyzer or special *h*-tube (fig. 3) is attached to a manometer. The 2 parts are on a base which can be tilted and fixed in any desired position. The lower cup on stand contains alkali which by Hg pressure can be forced into the side-arm of analyzer.

pleted. When this result is reached, the reagent is withdrawn through the tail-cock of the side-arm. The procedure will vary according to the pressure within the apparatus. If it is negative, the reagent tube is connected with the tip of the tail-cock as before, and then on lowering the cup the reagent is slowly withdrawn from the analyzer. When the pressure is positive, it is sufficient to open the tail-cock carefully, and collect the reagent in a small dish.

Because of the viscosity of the pyrogallate solution, it does not drain quickly. Moreover, the strong alkali tends to decrease the aqueous tension in the apparatus. To obviate these disturbing factors, it is advisable, as the next step, to introduce distilled water into the arm of the analyzer, following the same procedure as in the case of the reagent. After half an hour, the water can be removed and the manometric reading made.

It will be seen from table 2 that the oxygen was practically all removed in 3 hours. The subsequent slow rise in negative pressure is probably due to a decreasing aqueous tension under the influence of the strong alkali. The treatment with distilled water restores 3 to 7 mm. of aqueous tension.

The corrected manometric value obtained shows a marked agreement with the calculated figure. It is evident that the method can give values which are accurate to within 1 mm. of pressure. No claim can be made

TABLE 2
TRIPPLICATE MANOMETRIC ANALYSIS OF AIR

Tube No.		1	2	3	
Manometer No.		28	29	30	
Pyrogallate in.....	Min.				
	0	+38	+50	+50	
	26	—23	—18	—4	
	40	—60	—56	—52	
	60	74	73	70	
	80	93	88	82	
	100	102	97	95	
	120	104	98	100	
	140	109	101	101	
	160	110	103	103	
	180	113	104	105	
	200	115	105	105	
	220	116	106	107	
	300	—119	—107	—109	
Pyrogallate out.....		—153	—148	—153	
Distilled water in.....	0	—88	—78	—95	Barometer.....745
	30	—84	—76	—93	Aq. tension at 35.5..... 38.8
Distilled water out.....		—146	—145	—146	B - T.....= 706.2
Corrected man.		—148.0	—147.1	—148.2	Hence calculated manometer after removal of CO ₂ + O ₂ = 706.2 × 20.96 = 148.1 mm.
Calc. CO ₂ + O ₂		20.957	20.83	20.985	Air = 20.96

for rapidity of analysis. In this respect, the method is greatly inferior to the buret procedure. On the other hand, it has the advantage that the gas content of a culture can be determined without much special apparatus and experience.

Analysis of Aerobic Cultures.—The application of the manometric method in the analysis of an aerobic culture will be seen best from an example.

As before, a pledget of cotton was placed in the narrow arm. The solid stopper was removed and wrapped in paper and a cotton plug was inserted in its place. After sterilizing in the dry heat oven at 200 degrees,

10 c.c. of 2% plain agar were placed in the main arm, and the whole auto-claved at 110 degrees for 20 minutes. The tube was then slanted for some hours, after which the entire surface was inoculated with a loopful of a culture of *B. coli*, J. The cotton plug was then cut off and pushed within the main tube. The stop-cocks were then greased and turned firmly in place, and finally secured with rubber bands. The tube was then attached to the manometer by means of a No. 25 rubber stopper and the hose clamps. The two parts were then placed on the rocking stand and transferred to the hot-room, and finally equilibrated at the end of 2 hours.

The results of a triplicate experiment by the manometric method are given in table 3. The manometric reading, before analysis, was noted ($= b$).

TABLE 3

TRIPPLICATE MANOMETRIC ANALYSIS OF AN AEROBIC CULTURE OF *B. COLI*, J., PLAIN AGAR

Tube No.	1	2	3		
Manometer No.	29	28	30		
Equilibrated....	Hrs.			Temp.	Barometer 740
	0	0	0	33.5	
	2	0	0	.0	
	3	-1	-1	.4	
	4	-2	-6	.4	
	6	3	7	.4	
	8	3	9	.9	
	20	12	20	.1	
	32	22	30	.6	
	45	22	32	.4	
	49	-22	..	.4	B - T = 701.4
	70		29	.0	
	74	-29	..	.4	
	122		-26	.0	B - T = 702.26
Corr. Man. = b	22.317	29.403	26.397		
b^1	131.872	141.946	138.078		
b^2	146.074	149.043	147.215		
$\text{CO}_2 = b^1 - b$	109.555	112.543	111.681		
$\text{O}_2 = b^2 - b^1$	14.202	7.097	9.137		
Percentage CO_2	15.619	16.045	15.903		
Percentage O_2	2.025	1.012	1.201		
Percentage loss (b).....	3.182	4.192	3.759		
	20.826	21.249	20.963	Air = 10.96	
Percentage CO_2 gain.....	15.589	16.015	15.873	CO ₂ found less 0.03	
Percentage O_2 loss.....	18.905	19.918	19.629	20.93 less O ₂ found	
Real resp. quot.	0.824	0.804	0.809	Not corrected for dissolved CO ₂	

Then the KOH reagent was introduced into the analyzer, which was frequently rocked, and kept in a slanted position. The CO_2 was practically all removed within 1 hour, since the manometer changed but 3 mm. in the next hour. The reagent was then withdrawn and the manometer was read ($= b^1$). The pyrogallate solution was then introduced and allowed to act until the manometric reading was constant, which took place in $\frac{1}{2}$ - $\frac{3}{4}$ hour. It was then withdrawn, and the manometric reading was noted ($= b^2$). In this experiment, the residual pyrogallate was not washed out with water.

The manometric readings b , b^1 and b^2 are then corrected. The value b represents the real loss. The CO_2 tension $= b^1 - b$, while that of $\text{O}_2 = b^2 - b^1$. The O_2 consumed is given directly by b^1 .

The percentages are deduced as follows:

$$\begin{aligned}\text{Loss} &= \frac{b \times 100}{B - T} \\ \text{CO}_2 &= \frac{(b^1 - b) \times 100}{B - T} \\ \text{O}_2 &= \frac{(b^2 - b^1) \times 100}{B - T}\end{aligned}$$

Since pure air contains 0.03% CO₂ and 20.93% O₂, the actual gain in CO₂, and loss of O₂ can be arrived at. From these values the real respiratory quotient can be calculated.

$$\text{Resp. quot.} = \frac{\text{CO}_2 \text{ gain}}{\text{O}_2 \text{ loss}}$$

It should be pointed out, perhaps, that the medium dissolves some CO₂, which, if it were taken into account, would increase the value of the quotient.

Analysis of Anaerobic Cultures.—For this purpose, the culture tube, after inoculation with *B. coli*, etc., is attached to the manometer, evacuated and filled with pure N₂ or H₂. There is no loss of H₂ by diffusion or leakage, as is indicated by the fact that the apparatus, filled with H₂ under positive pressure, when kept at 34 degrees for 3 days, shows no change in the manometric reading.

The apparatus after having been filled with either of the gases mentioned is placed in the hot-room for from 1 to 2 hours, and is then equilibrated, the tip of cock 3 being under water to prevent air from entering. Cocks 1 and 3 are then closed. A positive pressure soon begins to develop, and in 48 hours it will exceed +100 mm. (= *b*). The alkali absorbent is then forced into the side-arm of the analyzer. Absorption of the CO₂ is completed in less than an hour. The alkali is then withdrawn and the manometer read (= *b*¹). This value is assumed to represent the tension of H₂.

$$\begin{aligned}\text{CO}_2 &= b - b^1 \\ \text{H}_2 &= b^1\end{aligned}$$

From the corrected values, the percentage of CO₂ and H₂ can be calculated, since B—T is known. The hydrogen quotient = $\frac{\text{mm. or per cent. CO}_2}{\text{mm. or per cent. H}_2}$. As mentioned above, the medium may dissolve considerable CO₂, especially if NH₃, etc., is made by the organism, and hence the foregoing quotient is lower than it should be.

The direct estimation of H₂ by absorption could be made by means of the reagent of Paal and Hartmann (1910), which is a colloidal solution of platinum with sodium picrate. There is, however, some question as to the accuracy of such procedure.

BURET ANALYSIS

The Henderson ⁴² modification of Haldane's gas apparatus ⁴³ was used in this work and proved to be satisfactory. It was found desirable, however, to modify it by the addition of a combustion chamber in which

⁴² Jour. Biol. Chem., 1918, 33, pp. 31-38.

⁴³ Methods of Air Analysis, London, Ed. 3, Griffin and Co., 1920.

H₂, or hydrocarbons, if any, could be burned by means of a heated platinum spiral. For this purpose a 3-way **T** glass cock, with 3 capillary arms, was attached so as to be over the KOH container, and the descending arm was fused to the capillary of the bell. The right arm was connected by rubber tubing with the capillary of the combustion chamber. The latter measured about 25 x 90 mm. and was closed by a 3-holed rubber stopper which was securely wired in place. Two of the holes received the glass tubing which carried the electric wires; a glass tube in the third opening connected with the Hg reservoir.

Certain additional features were added to the apparatus in order to facilitate the work of sampling and of analysis. These will be found in the general description which follows. The apparatus as a whole is seen in fig. 11.

The stop-cocks on the analyzer, like those on the manometer, should be firmly held in place by good rubber bands. Since the buret cock has a 4-way channeling, it is advisable to mark it so as to indicate the direction of the flow of the air. The lower half of the handle on the stopper, the buret being open, may be etched or covered with asphalt.

Calibration.—After having temporarily fused a glass stop-cock, with fine tip, to the lower end of the buret, it is then filled with Hg and carefully calibrated. The Hg is drawn to the 7 c.c. line and weighed; it is then refilled and the Hg drawn to the 8 c.c. line and weighed; this operation is repeated from 0 to 9 c.c. and 0 to 10 c.c. As a check, the portions from 7-8 c.c., 8-9 and 9-10 c.c. can be weighed separately. These weights divided by the density of the Hg, corresponding to the temperature at the time of calibration, give the correct volumes. A convenient table of values can then be drawn up.

It is a distinct advantage to have the upper part of the buret, the portion between the stop-cock and the bulb, of about the same bore as the lower portion and graduated to +1.0 c.c. This facilitates the analysis of tank gases, such as CO₂, N₂, or O₂, which contain less than 10% impurity. Moreover, it enables the direct analysis of the gas in a culture tube or jar when it contains 90% or more of O₂ or CO₂.

Distilled mercury is used in the apparatus, and whenever it becomes fouled it should be drawn off, the buret cleaned and refilled with fresh Hg. The inner walls of the buret are kept moistened with a drop of 1% sulphuric acid, to provide the requisite aqueous tension. Any excess of acid can be removed by a filter paper, after having raised the Hg level almost to the tip of the buret. Some of this acid solution is also placed in the control tube as indicated later.

Reagents.—For the oxygen absorptions, the reagent was prepared according to the formula given by Benedict.⁴⁴ It is advisable to keep a stock of the solution on hand in a rubber stoppered bottle. This allows any precipitate which forms to sediment; moreover, absorption is more rapid perhaps with an

⁴⁴ Carnegie Inst. of Washington, Publication 166, 1912, p. 80.

old mixture than with one freshly prepared. The stock solution is made by dissolving 45 grams of pyrogalllic acid in 45 c.c. of hot distilled water, and then adding 405 c.c. of KOH (sp. g. 1.55). The latter solution is prepared by dissolving 800 grams of the stick KOH, not purified by alcohol, in 400 c.c. of water. Since the water content of stick potash varies, it may be necessary

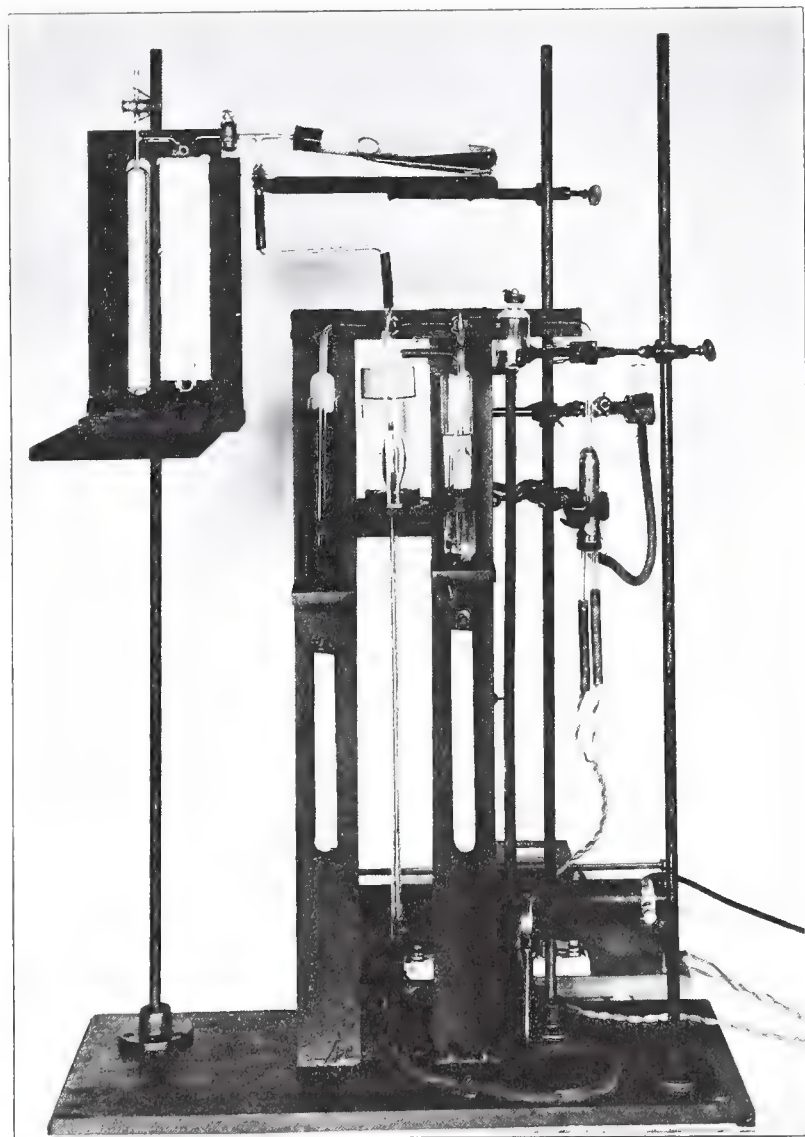


Fig. 11.—The modified Henderson-Haldane apparatus on stand with adjustable platforms to hold the manometer and culture tube or Novy jar. The gas sample is drawn from cock 3 on the manometer, through the capillary connector, into the buret.

to add more KOH to bring the solution to the desired density. About 60 c.c. of the pyrogallate solution are introduced into the test-tube through a bent thistle-tube, and the reagent is then covered with a layer of paraffin oil, about 30 mm. deep.

The CO_2 absorption is effected by means of 10% KOH, about 75 c.c. of which are placed in the other test-tube. It is advisable to cover the solution with a thin layer of paraffin oil, about 5 mm. deep, to prevent evaporation and to avoid the troublesome meniscus when adjusting the pressure level.

Stand.—We have found it useful to cut a long slot (2 x 39 cm.) in the support back of each absorbing tube. This facilitates the adjusting of the liquid to the line etched on the capillary tube. It also enables the use of a movable support for the tube which is loosely held by a ring band. Any friction present can be reduced greatly by paraffining the inner face of the slot.

The stand has been further improved by facing the slot with strips of angle brass between which the support operates. The latter with its tube is raised or lowered by means of a rack and pinion. This enables quick and accurate adjustment of levels, and also permits rapid transfer, back and forth, of the gas.

The solid frame carrying the apparatus is mounted on a large board (26 x 67 cm.) which carries 3 vertical rods (1.2 x 105 cm.). One of these, by means of its clamps, serves to hold in position the combustion chamber, and also carries its Hg cup. The Hg bottle of the buret is held in a long-armed clamp (18 cm.), which slides easily on the second rod and permits very quick adjustment of the Hg levels. It is not necessary to clamp it tightly to the rod, since a slight slant due to the weight of the Hg acts as an effective brake. The third rod holds an adjustable platform (15 x 25 cm.) on which the manometer or other container is placed when a sample of the gas is to be drawn for analysis. A similar platform is attached to the first rod and serves to support the culture tube, jar, etc.

The apparatus can be used in the main laboratory, but when a large number of analyses are being made, it is convenient to keep it in the hot-room at a nearly constant temperature of about 36 degrees. The gas samples can thus be drawn and analyzed with the least possible delay. At this temperature, the absorption of the gases proceeds rapidly.

Control Tube.—The arm of the control tube should be about 15 mm. shorter than the bell on the right arm of the buret, especially if the apparatus is used but occasionally, or when it is kept in a room where the temperature is subject to a considerable drop during the night. In the latter case, alkali can be drawn over into the control tube, and similarly it may be drawn over into the buret unless the 3-way cock above is closed. By lowering the KOH test-tube, the end of the control tube can be opened to the air. All of this difficulty, however, can be avoided by making the arm of the control tube in the form of a **T**, the outer branch of which is terminated by a stop-cock, as in the original Haldane apparatus.

The control tube and the 2 arms of the buret, as well as the capillary above the combustion chamber, should have an etched line on the same level, about 25 mm. above the bell.

If the lower end of the control tube is open and the short end is immersed in the KOH solution, so that the etched line coincides with the level within the capillary, it will be seen that the surface of the alkali in the large tube is lower, for example by 6.9 mm., than the former. Hence this capillary rise, which varies with the caliber and barometric pressure, should be taken into consideration. True atmospheric pressure within the control tube is therefore indicated when the line coincides with the level in the capillary and is ± 6.9 mm. above the meniscus of the alkali in the tube. This distance is determined by means of vernier calipers.

The capillary rise having been determined, the next step is to introduce 1% H_2SO_4 into the lower end of the control tube. For this purpose, a short piece of rubber tubing (100 mm.) is attached to the end of the tube, and by means of a thistle-tube, or otherwise, the dilute acid is introduced until the level corresponds to about that of the 8 c.c. line on the buret. A broad clamp is then applied to the rubber tube, after which the lower end is closed with a glass rod (75 mm.). By pushing in the glass rod, any air present in the tube can be forced upward. The filling of the control tube is easily and quickly done when the side-arm is provided with a glass-cock, as mentioned above.

To bring about atmospheric pressure in the closed control tube, the KOH tube is raised or lowered till the meniscus of the alkali is about 6.9 mm. below the line on the control tube. The level of the KOH in the capillary is then brought to the line by means of the clamp or the glass rod. A slight additional readjustment is necessary in order to obtain the exact capillary height with the liquid in the capillary on the line.

The subsequent adjustment of the pressure volume, before taking a reading on the buret, is made by raising or lowering the KOH tube until the capillary meniscus is brought back to the line.

Instead of having a single line on each of the arms, as mentioned above, it is preferable to have a space of 1 cm. graduated in mms. With such a scale provided on each arm, on opening the cock in the control tube and removing the buret cock, the capillary rise in the 3 arms can be compared. Any inequality in the level of the zero lines can thus be ascertained and noted.

In this case, to adjust the control tube, the KOH tube should be raised or lowered till the level of the alkali in the capillary is on the zero line. The cock is then closed. The control tube now contains the normal pressure volume. The cock remains closed during the analysis. Before taking a reading on the buret, the capillary level in the control tube must be brought back to the line by either raising or lowering the alkali tube.

Similarly, the line which coincides with the meniscus of the alkali in the arm of the buret, when the stopper of the latter is removed, is that to which all subsequent adjustments are made. To measure a gas, therefore, the level of the control tube having been adjusted, that of the KOH arm is brought to its line by raising or lowering the Hg bulb of the buret.

While the buret stopper is out, the level of the liquid in the capillary of the pyrogallate tube is likewise adjusted to its line. This is done by raising or lowering the pyrogallate tube. The distance between the levels of the pyrogallate, in the tube and in the capillary, represents the capillary rise, and it should be restored before the adjustment is made of the control tube or of the KOH arm for the purpose of reading the buret.

Assuming that the volume of the gas in the buret is the same as that in the control tube, it follows that any contraction or expansion that takes place is the same in both. Hence, bringing back the air in the control tube to its original pressure volume results in a like correction for the volume in the buret. The volume of the gas in the buret, however, changes as a result of the removal of the CO_2 and O_2 . Hence, it is desirable to begin the analysis with about 10 c.c. of the gas in the buret, and with about 8 c.c. of air in the control tube. The residual volume, after the absorption of CO_2 and O_2 will then approximate that in the control tube, and any error in expansion or contraction, due to inequality of volume, will be slight.

Filling with Nitrogen.—The apparatus having been set up, the next step is to fill the buret and arms with pure N_2 , which is obtained from air after

removal of the CO_2 and O_2 . The term nitrogen, of course, includes the argon which is present. The method of procedure is the same as that followed in a real analysis, and for that reason it may be given at some length.

About 10 cc. of air are drawn into the buret and the cock closed by a $\frac{1}{8}$ th turn. The Hg in the bottle is then placed on about the same level as that in the buret, after which the cock is turned to communicate with the KOH tube. The adjustment is now made of the levels in the control tube, the combustion arm, the KOH tube, and lastly that in the pyrogallate arm. The stop-cock is then turned back to connect with the KOH arm, and readjustment of level is made, if necessary; the same is done with the Hg arm. The levels in the KOH and pyrogallate are again checked to make sure that the pressure levels are the same. Having made the final readjustment in the KOH arm, the volume of the gas is read on the buret.

The gas is then passed repeatedly into the KOH arm to absorb the CO_2 present. Any CO_2 in the Hg arm is washed out by passing the gas over into the combustion chamber, then back into the buret, and thence into the KOH tube. After several washings of this kind, all of the CO_2 is absorbed. The volume of the remaining gas is read after having checked or readjusted the levels in the control tube and KOH arm. Any loss in volume is due to CO_2 .

The gas is then passed into the pyrogallate tube, and after 3 minutes it is returned to the buret. This operation is repeated several times until the volume becomes constant. It is simplified by raising and lowering the support with the pyrogallate tube by means of the rack and pinion. The next step is repeatedly to wash the oxygen out of the KOH and combustion arms, returning the gas each time to the pyrogallate. The operation is completed when the volume shows no further decrease, indicating that all of the O_2 has been removed. After adjusting the levels, the volume of remaining gas can be read, and the amount of oxygen obtained.

The nitrogen thus prepared, likewise that remaining after an analysis, is stored over pyrogallate, until a new analysis is to be made, when it is returned to the buret. The levels in the control tube and arms are then adjusted anew.

Drawing of Sample.—The gas to be analyzed, when in a culture tube, is drawn directly into the buret, the arms of which are filled with nitrogen. For this purpose, the manometer with the attached tube is placed on the platform at such height that the lower end of cock 3 is about 7.5 cm. above the tip of the buret. The end of the culture tube is rested on the other platform to avoid any undue stress on the manometer. In work with cultures sealed with sealing-wax, as a precaution against leakage, a Hg seal was applied at this point (fig. 12). The Hg seal, however, is unnecessary if the tube is actually and completely closed.

The buret and arms having pure N_2 , and the levels in the control tube and in the absorbing arms having been adjusted, the tip of the buret is then connected with the lower end of cock 3 by means of a bridge of capillary tubing, the ends of which are bent at right angles and in opposite direction as shown in fig. 11. The rubber tubing used to make the connections should be moistened with water. As far as possible, glass to glass connections are always made; at the same time, stop-cock 2 on the manometer is closed to prevent the Hg from being pulled out of the manometer when the sample is drawn.

On opening the buret cock and raising the Hg bottle, the Hg passes through the bridge connection and out of tail-cock 3, thus expelling all the air along

the line and establishing a perfect Hg connection with the culture tube. A small aluminum cup suspended over the end of the tail-cock is useful to catch the overflow of Hg. Now, on opening cock 3 and lowering the Hg bottle, gas is drawn into the buret. In order to insure a thoroughly mixed sample, the gas is then returned to the culture tube and again drawn out, and this is repeated several times. If a high negative pressure is in the culture tube, on opening cock 3, some Hg is likely to be drawn into the tube. This can be avoided by closing the cock by a $\frac{1}{8}$ th turn, and then lowering the Hg bottle to produce a negative pressure corresponding somewhat to that in the culture tube.

Finally, the gas is drawn into the buret so as to have as nearly 10 cc. as possible. The buret cock is then given a $\frac{1}{8}$ th turn, and the gas is thus shut off completely from the culture tube as well as from the alkali arm.

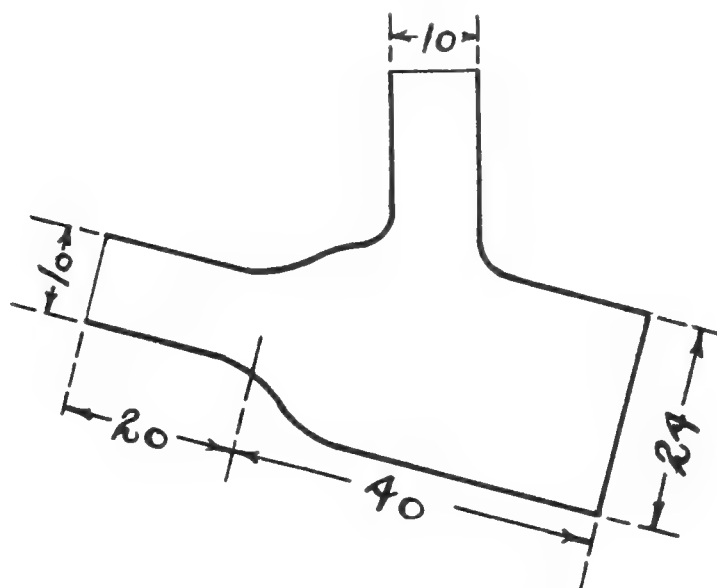


Fig. 12.—Mercury seal for culture tubes.

At the same time, cock 3 is closed. The Hg in the bottle is now brought to approximately the level of the Hg in the buret, and the gas is allowed to remain thus shut off for about 15 minutes, if in the main laboratory, in order that it may cool to the temperature of the buret. When the sample is drawn in the hot-room only a few minutes are necessary for this adjustment. Just before taking a reading, the water in the jacket is mixed by means of a bulb blown on the end of a piece of capillary tubing.

Having cooled the gas and agitated the water, connection is now established with the alkali arm by completing the quarter turn of the cock; the levels are then readjusted, and the volume of the gas is read off. A second or check reading should be made. The analysis can then be continued (see CO_2 Estimation).

For some purposes it is desirable to analyze the air in the test-tube, flask, or bottle cultures which are not attached to manometers. In such case, the tube or other container, after inoculation of the medium, is closed with a glycerolated perforated rubber stopper which carries a tube suitably bent and

provided with a tail-cock (fig. 13). When development has taken place, the container is placed on the platform and connected with the buret in the manner described, and the desired sample can then be drawn. The sampler (fig. 14) can be used to obtain a larger quantity of the gas for duplicate analyses.

The anaerobic jar, when unconnected with the manometer, can be readily sampled. Of the several methods which were tried, the following is the simplest and most accurate. The first step in the operation is to fill both ends of the cock on the jar with water, by means of a finely drawn, capillary pipet, which is bent at an angle. A short glass rod is inserted into a piece of rubber tubing (3 cm.), and the latter, after being filled with water, is

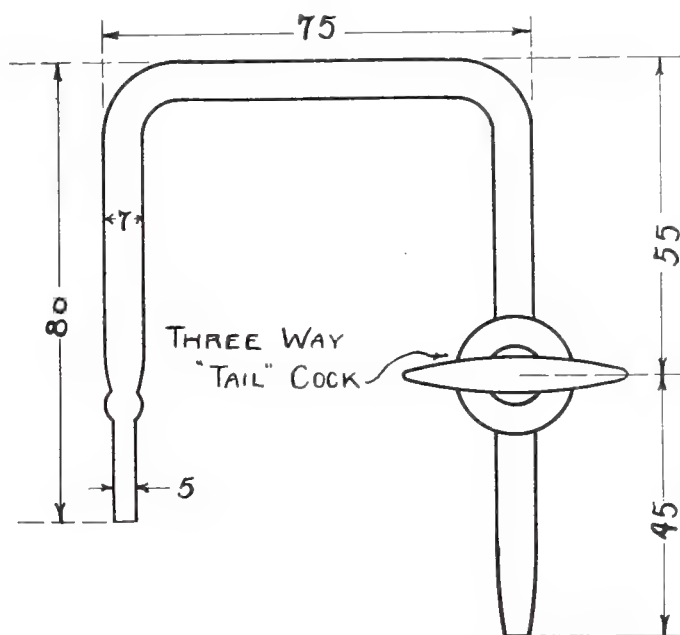


Fig. 13.—Three-way tail-cock for drawing a sample of gas from the tube or bottle. It is inserted at narrow end into a rubber stopper which fits the container.

carefully slipped over the head end of the jar-cock, while, at the same time, the glass rod is slightly withdrawn to release the pressure within the tube.

The jar is then placed on the left platform of the buret stand (fig. 11) at such a height that the tail end of the jar-cock, directed to the left, is on line with the tip of the sampler (fig. 14), which is on the table. A short piece of glass tubing, bent at a right angle, is attached to the tip of the sampler, its free end dipping into some water in a small beaker or flask. About 10 c.c. of water are then drawn into the sampler, and the bent tube is disconnected.

A piece of wet rubber tubing is now attached to each end of the connector, which, in this case, is a straight piece of capillary tubing (14 cm.). One end is now slipped over the tip of the sampler, making a glass to glass joint. The Hg bulb is now raised, to expel the air, and to fill the connector and the

rubber tube on the end with water. The end piece of rubber tubing is then slipped over the tail end of the jar-cock, thus establishing a perfect air-free connection.

The Hg bulb is then lowered so as to create in the sampler a negative pressure of about 100 mm. Holding firmly the rubber over the tail end of the cock, the latter is rotated so as to open up connection with the interior of the jar. The water in the connection, and some gas, now passes over into the sampler because of its negative pressure. Whereupon the Hg bulb is raised until the water reaches the tail-cock on the sampler. This cock is then turned so as to allow the water to flow out of the tail. As soon as the Hg reaches the cock, the latter is turned back and the Hg bulb is lowered, when nothing but gas is drawn into the sampler. It is well to send the gas back and forth several times, in order to secure a good sample. Finally, about 30 c.c. of the gas are drawn. Then the rubber tube over the end of the jar-cock is held as before while the cock is rotated back into its original position, thus closing the jar. Lastly, the tail-cock on the sampler is turned so as to admit air into the connector, after which the latter is disconnected. The transfer of the gas from the sampler to the buret is effected in the manner described below.

Sampler.—When the anaerobic bottle or jar is connected with a manometer, the combined apparatus can also be placed on the two platforms and the sample drawn directly into the buret as described above for a tube culture. It is more convenient, however, to make use of the sampler shown in fig. 14. The body of the sampler is 1 cm. in diameter and about 50 cm. in length. It is graduated in 0.1 c.c. and has a capacity of 30 c.c. The lower end is narrowed to about 7 mm. and connects with a Hg bottle. The upper end has a 7 mm. capillary, which is bent at right angles and carries a tail-cock. This end is connected by means of a capillary L with the tip of cock 3 on the manometer. At the same time, cock 2 is closed. On raising the Hg bottle, the air is expelled from the sampler and the connector, and finally Hg begins to drop from cock 3. The latter is then closed by a $\frac{1}{8}$ th turn, and the Hg bulb is lowered somewhat to insure a negative pressure, after which the $\frac{1}{4}$ turn is completed. The air sample is now withdrawn, and returned repeatedly to obtain a perfect mixture, after which 20-30 c.c. of the gas are drawn into the sampler, the tail-cock of which is then closed by a $\frac{1}{8}$ th turn. Cock 3 on the manometer is then closed, and the $\frac{1}{4}$ turn of the cock on the sampler is completed.

It is not possible to make a very satisfactory check or duplicate analyses of the gas within a culture tube, for the reason that the decrease in pressure, following the withdrawal of the first sample, causes liberation of some of the dissolved CO_2 . Moreover, an actively growing organism may produce appreciable changes in the gas content in an hour or less. Hence, when it is desired to control the analytic method by duplicate analyses it is preferable to use the sampler referred to above. In that case, 20-30 c.c. of the gas from the culture tube or jar are drawn into the sampler, and from it such portions as needed are transferred to the buret.

The next step is to transfer the gas from the sampler to the buret. To do this the sampler is placed on the lowered platform, and the L connector is rotated and attached to the top of the gas buret. The Hg from the latter is then passed through the connector into the 3-way cock, which is then turned, and the gas is drawn into the buret. The cock on the latter is then closed

by a $\frac{1}{8}$ th turn, after which the cock on the sampler is closed, and the buret is disconnected.

The Bailey⁴⁵ bottle or sampler was found to be very useful in drawing specimens of the outside air, or that within the laboratory, or in the hot-room. It was likewise convenient in sampling the CO_2 , N_2 and O_2 contained in pressure tanks or to store pure N_2 or O_2 . To transfer the gas, the Bailey

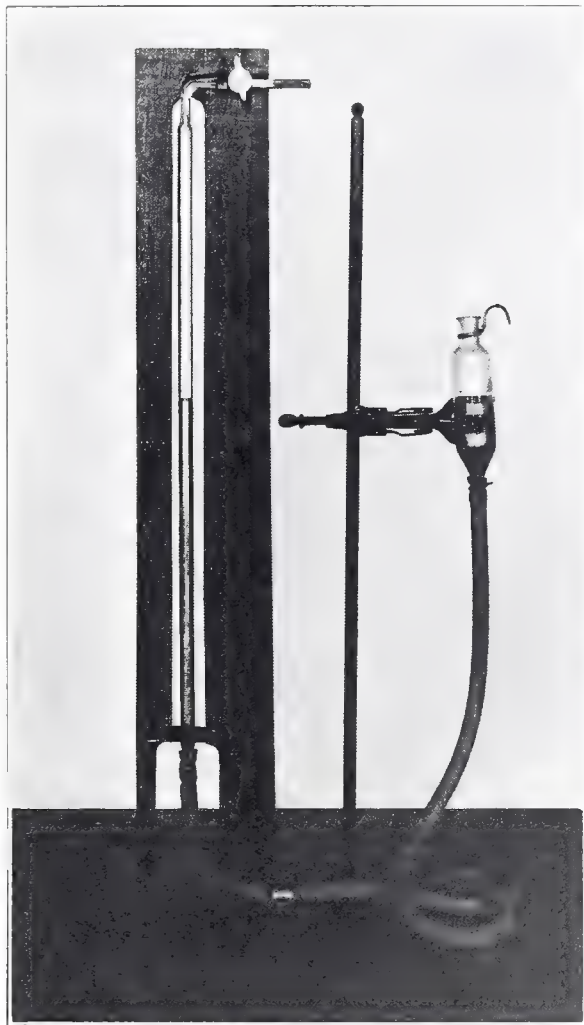


Fig. 14.—Sampler filled with Hg, used to withdraw gas from anaerobe jar or other container.

bottle was placed on the platform, and the L connector attached to it and to the tip of the buret; the air in the connector was then expelled, and the transfer effected in the manner just described.

Carbonic Acid Estimation.—Having measured the volume of the gas to be analyzed, the next step is to determine the amount of CO_2 present. The gas

⁴⁵ Jour. Biol. Chem., 1921, 47, pp. 281-283.

should be passed slowly into the KOH arm, especially if the amount of CO_2 is large. It is then sent back and forth about 10 times, and readings are made after the usual adjustment of levels. When two such readings agree, it shows that all the CO_2 is removed. The difference between this and the original volume gives the uncorrected volume of CO_2 .

Oxygen Estimation.—The gas is then passed into the pyrogallate to absorb the O_2 , after which the KOH arm is washed out in the manner given. Frequent raising and lowering of the support with the pyrogallate tube, or of the Hg bulb, will greatly increase the speed of absorption. Readings are taken, as before, until they agree, indicating that all of the oxygen has been absorbed. The difference between this reading and that after removal of CO_2 represents the uncorrected volume of O_2 (see H_2 Estimation).

When the total of $\text{CO}_2 + \text{O}_2$ exceeds 30%, it is customary to make the analysis with a smaller sample. For this purpose, a definite amount of N_2 , for example some of the reserve N_2 , is carefully expelled so as to leave in the buret about 7.2 c.c. This is then accurately measured and stored in the pyrogallate arm. Then about 2.5 c.c. of the gas to be analyzed are drawn into the buret. The two gases are then combined, and the volume is read. The difference between this and the original volume of the N_2 represents the volume of the gas to be analyzed. The CO_2 and O_2 determinations are then carried out in the usual way. Obviously, the analysis of such a small sample tends to increase the error, if any, about 4-fold. The error is especially in evidence when calculating the respiratory quotient of an organism grown in high O_2 tension (table 4). In such case, any error in determining the small amounts of nitrogen present is reflected in the nitrogen factor which may thus be extremely low.

It not infrequently happens, starting out with a 10 c.c. sample, which yields perhaps 28% of CO_2 that, after the O_2 is absorbed, the residual volume is something less than 7 c.c. Hence the actual amount cannot be read off on the buret. Instead of discarding the analysis and repeating it with a smaller sample, by the procedure outlined above, a satisfactory estimation of the residual volume can be made in the following way. After carefully adjusting the levels, the same as if a reading were to be taken, the gas is transferred to the pyrogallate arm. Then about 10 c.c. of air is drawn into the buret and sent back and forth into the KOH arm to remove any CO_2 present. The volume of the air is then measured in the usual way, and an exact amount, for example 2.8 c.c., is transferred into the KOH arm, while the rest of the air is expelled from the buret. The gas in the two arms is then combined and measured. The difference between this total volume and 2.8 c.c. gives the residual nitrogen, and now the O_2 volume can be deduced.

A more accurate procedure would be, after storing the gas in the pyrogallate arm, to draw about 10 c.c. of pure N_2 into the buret. After measuring the volume, about 3 c.c. are sent over into the pyrogallate arm, and the remaining volume is again measured, the difference being the exact volume of N_2 added.

This method can be applied whenever the residual N_2 volume is greater than 4 c.c. If it is less than 3 c.c., it can also be read indirectly by drawing into the buret 7 c.c. of air or N_2 .

It has also happened, that with a 10 c.c. sample, after absorption of CO_2 , the residual volume could not be read. In such case, the gas is transferred into the KOH arm, and pure N_2 is drawn from a Bailey bottle into the buret,

and the volume measured as accurately as possible. Then 3 c.c. of the N_2 is transferred to the pyrogallate arm, and the remainder is expelled from the buret. On combining the gas from the two arms, the volume can now be ascertained, and the difference gives the CO_2 reading. If N_2 is not available, ordinary air can be drawn and 3 c.c. transferred into the KOH arm. In the subsequent O_2 determination, the additional O_2 thus introduced must be allowed for.

The accuracy of the apparatus and of the methods used should be controlled by analyses of the outside air. These should give close to 0.03% CO_2 and 20.93% of O_2 , the allowable error being $\pm .05\%$. The air in the hot-room on different occasions gave

$CO_2 = 0.08$	$O_2 = 20.95$
0.09	20.91
0.16	20.77
0.19	20.80

Hydrogen Estimation.—The next step is to determine the amount present, if any, of H_2 or other combustible gas. Since all of the O_2 has been removed, it becomes necessary to add a sufficient amount of this gas to insure complete combustion. The O_2 required for this purpose is drawn from a tank, through an alkali wash bottle, into a Bailey sampler, from which the desired amount is transferred to the buret. First of all, however, the gas present in the buret is stored in the pyrogallate arm. Then about 1 to 1.5 c.c. of O_2 is drawn into the buret from the Bailey bottle. The amount of O_2 to be taken depends on the volume of the remaining gas, since the total volume must not exceed 10 c.c., the limit of graduations. The gas to be analyzed is then transferred back to the buret; the levels are adjusted and the total mixed volume read off.

The gas is then passed into the combustion chamber and is ready for combustion. The necessary current was drawn from the lighting system, A. C., 220 volts, and passed through a 60 cycle transformer (bell-ringer) and a Beck Bros. rheostat. The platinum wire is gradually heated to a low red for a minute (Dennis⁴⁶). The gas is passed back and forth 10 times; then the current is shut off, and the gas is returned to the buret. After it has cooled, the levels are adjusted, and the volume is read. It is again transferred to the combustion tube and the burning carried on with the wire at a dull red heat, the gas again being passed back and forth 10 times. It is then returned to the buret, and after cooling and adjusting of levels the volume is read a second time.

If the two readings agree and show no loss when compared with the reading taken before combustion, it means that no hydrogen or hydrocarbon is present. On the other hand, a distinct loss indicates the presence of one, or the other, or both of these gases. In that case the gas is passed back and forth into the alkali tube to wash out the arm and to remove any CO_2 which may have been formed by the combustion. Finally, it is returned to the buret, and after adjustment of levels the volume is noted. No change in the volume indicates absence of CO_2 and, hence, absence of hydrocarbons in the original gas.

The gas which now contains any H_2 that was present in the KOH arm is then passed into the combustion chamber and burned as before, after which it is returned to the buret, measured, and transferred to the alkali, then brought back into the buret, and the volume determined in the usual way.

⁴⁶ Gas Analysis, New York, The MacMillan Company, 1913, p. 147.

There still remains some gas containing hydrogen in the capillary of the pyrogallate arm. This necessitates the removal of all of the O_2 , measuring the resulting volume, returning it to the pyrogallate and drawing in some fresh O_2 and proceeding as in the beginning.

Since 2 volumes of H_2 unite with 1 volume of O_2 , it follows, in the absence of hydrocarbons, that the loss in volume, due to combustion, when multiplied by $\frac{2}{3}$ gives the amount of hydrogen present.

Estimation of CO_2 in the Medium.—The analysis of the gas over the culture does not give the total gas change, since a considerable and varying amount of CO_2 is taken up by the medium. The CO_2 may be present in mere physical solution; or, reacting with Na_2CO_3, Na_2HPO_3, Na of proteins, etc., it may form $NaHCO_3$; or, combining with the NH_3 and amines made by microbic action, it may yield corresponding carbonates. Thus, a culture of the Hay bacillus, growing on 10 c.c. of plain agar, may produce enough alkali to bind 9 and even 15 c.c. of CO_2 .

Consequently, it may be stated that the amount of dissolved or fixed CO_2 is an indication or measure of alkali production, notably of NH_3 . The relation of the gaseous to the dissolved CO_2 is designated as the

$$CO_2 \text{ quotient which} = \frac{\text{c c. dissolved } CO_2}{\text{c c. gaseous } CO_2}$$

When working with broth cultures, Van Slyke's⁴⁷ apparatus for the determination of CO_2 in blood plasma can be used. Obviously, this method cannot be employed when working with solid mediums. Satisfactory determinations of the dissolved CO_2 can be made, in liquid or solid medium, by the following method.

Aeration Method.—This may be given as the method of choice. As soon as the sample of gas is drawn for analysis, the culture tube is detached from the manometer, the cotton plug is discarded, and 10 c.c. of paraffin oil are poured in so as to cover completely the agar surface. The tube, with the medium thus covered, is set in an inclined position in ice-water or in cracked ice. The next step is to remove the gaseous CO_2 by aspiration or by compressed air. A rubber stopper provided with an inflow and outflow tube is inserted into the mouth of the culture tube. Compressed air, after passing through a KOH wash bottle, is forced through the former. The outflow tube is bent downward and carries near the end a double perforated rubber stopper. After the air has been driven through the tube for about 5 minutes, a small bottle containing 5-10 c.c. of $Ba(OH)_2$ is attached to the stopper on the lower end of the outflow tube. This serves to indicate whether or not all of the gaseous CO_2 has been expelled from the tube.

When all of the gas has been expelled, 2 c.c. of H_2SO_4 (1:5) and 5-10 drops of caprylic alcohol are added to the iced culture tube, which is then closed with a double perforated stopper carrying an inflow and an outflow tube. This tube is then connected, in series, with 3 tubes containing N/10 $Ba(OH)_2$. The first tube has 10 c.c.; the second, 5 c.c. and the third 2 c.c. of the baryta solution, plus 5 and 8 c.c., respectively, of CO_2 -free water. When a very large amount of CO_2 is present, it is advisable to place 10 c.c. of the baryta solution in the third tube. Figure 15 shows the arrangement of the glass parts. Through

⁴⁷ Jour. Biol. Chem., 1917, 30, pp. 347-368.

the inflow tube, which extends to the bottom of the culture tube, compressed air is passed after going through KOH and H_2SO_4 , which are contained in Friedrich's spiral wash bottles.

The culture tube (c) is then placed in a special oval Roux water-bath, and the temperature is raised to about 90 C. As soon as the agar has melted, the temperature of the bath should be lowered to about 70 C. to avoid loss of the caprylic alcohol. The air is allowed to stream through the system at a rate of 50 or more bubbles per minute for from 1 to 2 hours.

The baryta tubes are then disconnected, stoppered, and placed in ice water. When cooled to 0 degrees, each tube, after receiving 3 drops of 0.1% solution of phenolphthalein, is titrated with N/10 H Cl to disappearance of the red

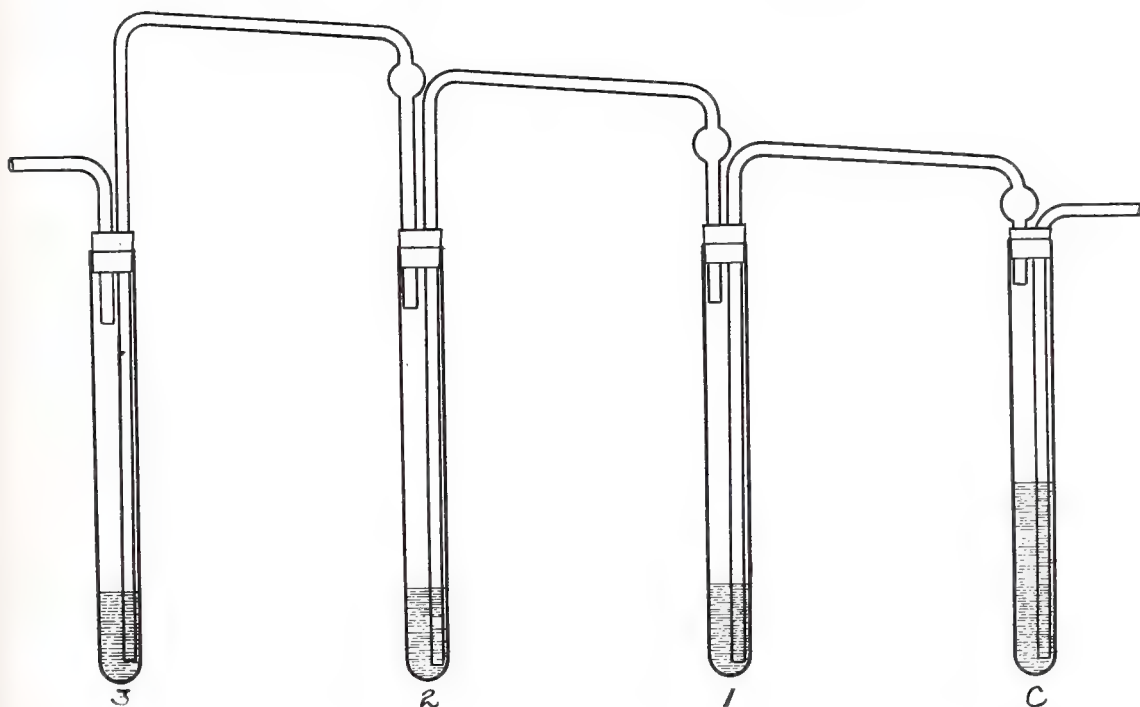


Fig. 15.—Aeration apparatus for the estimation of CO_2 taken up by the medium. The culture tube C is half immersed in a water-bath. Tubes 1, 2, 3 contain N/10 $\text{Ba}(\text{OH})_2$.

color. The method here given is a modification of that recommended by Truog.⁴⁸ The titration has an excellent end-point. The difference between the amount of baryta present at the beginning and at the end gives the amount neutralized by the CO_2 . The decinormal factor of CO_2 represents 2.2 mg., or 1.1129 c.c., CO_2 at 0 degrees and 760 mm. pressure.

The determination of the CO_2 content of a blood-agar medium requires a slight modification of the foregoing procedure. It is impossible to liquefy the medium, since, in the presence of agar, the blood coagulates at a relatively low temperature, thus making it difficult to secure proper aeration of the material. After the CO_2 over the paraffined, iced medium has been expelled, a clean glass rod with a flattened end is inserted, and the mass is comminuted

⁴⁸ Jour. Industrial & Engineering Chem., 1915, 7, pp. 1045-1049.

as finely as possible. The culture tube, after connecting up as indicated above, is placed in the water-bath, and the temperature is raised slowly to about 40 C., and kept there for an hour. The temperature is then gradually raised to about 60 C., and the air is allowed to stream through the system, at the rate of 50 or more bubbles per minute for about 3 hours.

Shaking Method.—This is less convenient than the foregoing, but gives fairly satisfactory results. The culture tube is treated as in the preceding method, but instead of being connected with the aeration system, it is joined to a tube of like diameter containing 10 c.c., or more, of N/10 Ba(OH)₂ by means of a U connector which has the same diameter as the tubes. A similar connector, it may be added, was described and used by Nichols and Schmitter⁴⁹ for the cultivation of anaerobes by the pyrogallate method.

The culture tube is then placed in the Roux water-bath, while the baryta tube remains on the outside. The tube is kept in the boiling water-bath for 2 hours. It must be taken out every 2 or 3 minutes and vigorously shaken to emulsify the oil and thus allow the CO₂ to escape from the melted agar. The baryta tube, after cooling to 0 degrees, is titrated as described in the foregoing.

The above methods give the total CO₂ present in the culture medium. From this amount is to be deducted the total CO₂ present in a control tube which is treated in like manner. Three control tubes of glycerol agar tested, side by side, by the aeration method gave 0.27, 0.30 and 0.31 c.c. of CO₂.

When a tube with water is kept in the respiration chamber, the amount of CO₂ which it takes up must also be determined. For this purpose, as soon as it is taken out, it is covered with a layer of oil, iced, and any CO₂ in the air of the tube expelled, as in the case with agar. A known amount of baryta can then be added, and the excess titrated directly with the N/10 HCl. At 32 C., 10 c.c. water may take up 0.67 c.c. CO₂ out of a gas containing 18% of CO₂ (table 5).

It is not possible to estimate by any heating method the free CO₂ which is merely dissolved in the agar at the temperature employed. On heating, any carbonate which may be present in the agar is largely dissociated into free CO₂ by the mass action of the phosphates and proteins. Thus, for example, if 0.5 c.c. N Na₂CO₃ is added to 10 c.c. of the agar medium, practically all of the CO₂ (5.56 c.c.) will be set free on prolonged heating although no H₂SO₄ is introduced. The same thing happens, though to a lesser degree, when agar, to which Na₂CO₃ is added, is sterilized in the autoclave.

An indication of the amount of CO₂ in simple solution in the medium can be obtained by placing in the container a test-tube with an equal volume of distilled water (CO₂-free). If the amount taken up by the agar medium is about the same as that in the water, it can be considered as being in plain solution (Part II, table 3). On the other hand, any appreciable excess is indicative of the formation of alkali.

Reduction of Volume.—Reduction of the volume of the gas present in the culture tube or jar to standard conditions, that is, to a dry state at 0 degrees and 760 mm. can be made by means of the usual formula:

$$V_0 = \frac{V}{1 + 0.003665 t^{\circ}} \cdot \frac{B \pm b - T}{760}$$

V = actual air space in the tube or jar, plus the space in the right arm of the manometer which was determined when the instrument was calibrated.

⁴⁹ Jour. Med. Res., 1906, 15, pp. 113-115 (Plate).

The method of obtaining the air volume in a jar has been given. Allowance must be made for the volume of water, agar and glass in the test-tubes which are in a jar. B = barometer at the start, or time of equilibration. b = corrected pressure as shown by the manometer or calculated from the analytical results. If negative, this is subtracted from B ; if positive, it is added. T = aqueous tension on the hydrogen scale at t degrees, the temperature at which the manometer is kept. The ordinary tables of aqueous tension do not go above 35 C. For temperature above 35 C., Van Nostrand's "Chemical Manual"⁵⁰ is useful.

INTERPRETATION OF ANALYTIC VALUES

The results as obtained by analysis must not be taken at their face value when final conclusions or comparisons are to be made. They represent apparent and not real values. The failure to perceive this difference is often conspicuous in the published studies on the gas exchange of organisms.

To evaluate the results of analysis it is necessary to have a control analysis of the gas content of the culture tube at the very beginning of the experiment. This expresses in percentage the amounts of CO_2 , O_2 and N_2 then present. As a matter of fact, the air in a culture tube undergoes an immediate change, and analysis gives values which differ from those of pure air. Hence, in general it is advisable to assume that pure air was present. With this assumption the initial air would have the composition: $\text{CO}_2 = 0.03$; $\text{O}_2 = 20.93$; $\text{N}_2 = 79.04$.

Assuming that the organism concerned does not fix or make nitrogen, it follows that the absolute amounts of CO_2 and of O_2 change, and even H_2 may be produced, while the actual amount of N_2 remains intact.

Analysis of the culture, however, will show that the percentage of N_2 has changed, though not to the same extent as is the case with the other two gases. If, at the end of the experiment, the pressure in the culture tube was the same as at the beginning, the percentage of N_2 would be the same as that of the control. But the percentage ordinarily is not the same for the reason that the pressure has changed. The pressure usually becomes negative because the absolute amount of $\text{CO}_2 + \text{O}_2$ has been decreased. Hence in such a case the N_2 shows an apparent increase although the absolute amount has not changed. It is clear, therefore, that the analytic results must be recalculated or reduced to the original nitrogen basis. The correction or nitrogen factor necessary for this is obtained from the formula:

$$\text{Nitrogen factor} = \frac{\% \text{N}_2 \text{ in control}}{\% \text{N}_2 \text{ in culture}}$$

⁵⁰ Ed. 4, edited by J. C. Olsen, New York, Van Nostrand, 1918, p. 533.

Multiplying the percentages found in the culture by this factor gives the corrected or real values.

The importance of this recalculation may be illustrated by an extreme example, that of a culture of the tubercle bacillus grown under high O₂ content. This was a very rich culture as the result of having been refilled with air 12 times in the preceding 46 days. For this test, it was evacuated and refilled with pure oxygen. It may be added that the tube (type 6) was sealed in the flame and was attached to the manometer by a No. 25 rubber stopper. The low value for the respiratory quotient

TABLE 4
A COMPARISON OF APPARENT AND REAL VALUES OBTAINED FROM A TUBE CULTURE OF
B. TUBERCULOSIS, GLYCEROL AGAR, 84 HOURS; BAROMETER, 740;
TEMPERATURE, 37.8 C.; B-T = 690.83

Analysis	Initial	Final	
CO ₂	0.69	86.65	Apparent gain CO ₂ = 85.96
O ₂	91.74	3.59	Apparent loss O ₂ = 88.15
CO ₂ + O ₂	92.43	90.24	Apparent loss = 2.19
N ₂	7.57	9.76	Calc. apparent manometer = -15.13 mm.
	100.00	100.00	Calc. apparent resp. quot. = 0.975
Corrected analysis*			
CO ₂		67.21	Real gain CO ₂ = 66.52
O ₂		2.78	Real loss O ₂ = 88.96
CO ₂ + O ₂		69.99	Real loss = 22.44
N ₂		7.57	Calc. real manometer = -155.02 mm.
		77.56	Corr. observed man. = -162 mm.
Loss.....		22.44	Calc. real resp. quot.† = 0.747

* Obtained by multiplying the above culture values by the nitrogen factor $\frac{7.57}{9.76} = 0.7756$

† Uncorrected for dissolved CO₂. Air volume in tube about 50 c.c.

indicates error due to fractional analyses or to absorption of CO₂ by the rubber stopper and the medium.

The analytic values for the control and culture are given in the upper half of table 4.

It will be noted that the apparent loss, based on the difference between the sum of CO₂ + O₂ at the start and that at the end of the experiment, is 2.19%. Multiplying this percentage loss by 690.83, or B - T, gives a calculated negative pressure of -15.13 mm., whereas the corrected observed manometric pressure was -162 mm.

The lower half of the table gives the corrected value for the culture. It will be seen that the real percentage of CO₂ is considerably less than the apparent value; further, that the real loss is 22.44% as against 2.19%

for the apparent loss. And, further, it is to be noted that the calculated manometric value is -155.02 , which corresponds closely to the corrected observed value of -162 mm.

It will be seen from the foregoing that the analytic data which add up to 100% give no direct evidence of the pressure conditions in the culture tube. When recalculated, however, to the nitrogen basis, the sum thus obtained indicates at once whether the pressure is negative or positive. The difference between it and 100 is an exact measure of the change in pressure. When the culture tube is filled with pure O_2 , obviously the nitrogen basis is not available for recalculations. The procedure to follow in such a case will be given later.

RESPIRATORY OR OXYGEN QUOTIENT

In the ordinary combustion of an organic substance, a definite volume of CO_2 is produced, and a definite volume of O_2 is consumed. The ratio of this exchange is expressed as a quotient, which is the same whether the volumes are expressed in c c. or as percentage, or as mm. of pressure.

$$\text{Resp. quotient} = \frac{\text{CO}_2 \text{ produced}}{\text{O}_2 \text{ consumed}}$$

Now, the theoretical respiratory or oxygen quotient for a given chemical compound can be deduced from the equation which represents its combustion. Thus, in the case of glycerol, we have the equation:



It follows therefore that the resp. quot. $= \frac{6 CO_2}{7 O_2} = \frac{6}{7} = 0.857$.

Similarly, the oxygen quotient for other organic substances can be deduced. The values thus arrived at may be seen in the following list.

Stearic acid	0.692	Acetic acid	1.0
Oleic acid	0.706	Alanine	1.0
Acetaldehyde	0.80	Formaldehyde	1.0
Butyric acid	0.80	Glucose	1.0
Leucine	0.80	Glycogen	1.0
Valine	0.833	Lactic acid	1.0
Glycerol	0.857	Pyruvic acid	1.2
Lysine	0.857	Glycine	1.33
Propionic acid	0.857	Formic acid	2.0

The combustion process which takes place in the living cell is not necessarily limited to the oxidation of but one compound. Further, it is not to be assumed that a given substance, for example glycerol, is completely burned to CO_2 and H_2O . One might therefore expect that a culture grown on glycerol medium would not give the exact glycerol

quotient, but rather a value which would be either higher or lower, depending on the composition of the other substances which are undergoing oxidation. Moreover, the mere fact that an organism is growing on a glycerol medium does not imply that it is oxidizing the glycerol present, though possibly this is the case. It is therefore of some importance to have exact determinations of the respiratory quotients for different organisms when grown on varied mediums. Hitherto, practically no accurate work of this kind has been done.

A common error has been the use of the uncorrected analytical values. The result in such instance is an apparent and not the real respiratory quotient. This may be illustrated in a striking manner by reference to table 4. Taking the apparent values as there given it will be seen that the

$$\begin{aligned}\text{Apparent resp. quot.} &= \frac{85.96}{88.15} = 0.975 \\ \text{Whereas, the real resp. quot.} &= \frac{66.52}{88.96} = 0.747\end{aligned}$$

The calculated real quotient in this case is considerably lower than the apparent one. This is due chiefly to the fact that the medium dissolved a relatively large amount of CO_2 .

The real respiratory quotient, as shown in table 4, requires the conversion of the found values into real values. This is done by multiplying the former by the nitrogen factor. The process of calculation may be summarized by the formula:

$$\begin{aligned}\text{Real resp. quot.} &= \frac{(\text{N}_{2f} \times a) - 0.03}{20.93 - (\text{N}_{2f} \times b)} \\ a &= \text{CO}_2 \% \text{ found} \\ b &= \text{O}_2 \% \text{ found} \\ \text{N}_{2f} &= \text{nitrogen factor} = \frac{\text{N}_2 \text{ of control}}{\text{N}_2 \text{ found}}\end{aligned}$$

But the real quotient in the foregoing example is not correct, since it does not take into consideration all of the CO_2 produced. The mistake of overlooking the CO_2 dissolved in the medium is a general one, largely due to the fact that there may be a little difficulty in estimating the amount of gas thus held. By means of the methods already given, it is possible to obtain a fairly accurate measure of this dissolved or fixed CO_2 . The amount of CO_2 is obtained in terms of gas at 0 degrees and 760 mm. pressure.

To arrive at the true amount of CO_2 produced, the net volume of the gas in the culture tube or jar should be reduced to 0 degrees and 760 mm.

pressure. Multiplying this reduced volume by the real gain in CO_2 , which is the difference between the corrected analytical value and the percentage of CO_2 present at the beginning of the experiment (control), gives the gaseous CO_2 in c. c. at 0 degrees and 760 mm. To this amount should be added the number of c. c. of CO_2 held by the medium, less the amount present in the control agar.

Similarly, the real O_2 loss is obtained by subtracting the corrected analytical value for O_2 from that present at the start, as shown by the control analysis. Multiplying this into the reduced volume gives the number of c. c. of O_2 (0 degrees and 760 mm.) consumed or lost. Dividing the total number of c. c. of CO_2 produced by the c. c. of O_2 consumed should give the correct real respiratory quotient.

The true real quotient should express the relation between the CO_2 output and the O_2 consumed by the cell. Secondary reactions may be expected which will affect the value of the quotient. Thus, when an organism gives rise to amines it necessarily liberates CO_2 by carboxylase action. The total of CO_2 produced is thereby increased, and hence the respiratory quotient will be higher. An example of this will be given presently.

Again, given a facultative anaerobic organism, it is possible that it will continue to produce CO_2 after the O_2 has disappeared. In such case, the quotient will be much higher than it was earlier in the experiment. Compared with this, the effect of secondary carboxylase action, mentioned above, is relatively much less. Hence, when a respiratory quotient is 2 or 5 or 10, it means that anaerobic respiration has followed the aerobic.

The respiration chamber may be a culture tube attached to the manometer (fig. 4). In the interest of accuracy, however, it is much better to employ the anaerobic bottle or jar connected to a manometer (figs. 5 and 6). The larger volume of air permits repeated sampling and lessens the analytic errors. With only one culture tube, containing 10 c. c. of agar, in a jar of 2,500 c. c. capacity or more, the dissolved CO_2 is almost negligible, provided no alkali is made by the organism.

With only one culture tube in the jar, the maximal gas change is not effected as speedily as when 2 or 3 tubes are used. It is desirable that the culture should use up the O_2 as quickly as possible in order to lessen any tendency to auto-oxidation of the medium. A secondary reaction of that kind would have the effect of lowering the value of the respiratory quotient.

The manometer is a fair indicator of the respiratory quotient. Thus, a negative pressure, as is obtained with ordinary agar cultures, shows that the quotient is less than 1. When glucose agar is used, the manometer may read zero or possibly $+2$ or 4 mm., thus indicating a quotient of approximately 1. A higher positive pressure would point to a quotient greater than 1.

The volume of the chamber must be determined, and allowance made for the amount of agar, water and glass of the tubes. The latter is arrived at by displacement. The culture tube, after it has been cleaned, is filled with water, the volume being noted, for example, 52 c c. It is then lowered into a graduate containing 30 c c. of water. The total volume now may be 91 c c. Hence, $91 - 52 = 39$ c c., or the volume of the glass in the tube.

In table 5 are presented, by way of illustration, the results of a determination of the respiratory quotient of *B. subtilis* when grown on 1% agar containing 5% glycerol.

An anaerobic jar received 2 inoculated tubes (20×150 mm.), very loosely plugged, each containing 10 c c. of the medium; also 1 open tube with 10 c c. of boiled distilled water, the latter to supply the requisite aqueous tension. In order to hasten the production of this tension, 5 drops of water were placed on the bottom of the jar. Thereupon the jar was closed, clamped, and attached to a manometer and placed in the hot-room at 32°C ., with stop-cocks 1 and 3 closed. It was equilibrated 4 hours later.

In 10 days, the manometer showed a pressure of -20 mm.; on the 13th day, it reached -21 , and remained at that point till the 19th day, when the content was analyzed. The corrected manometric reading was -21.6 mm. It will be seen from table 5 that the O_2 was practically all gone, and evidently this state was reached about the 10th day. The culture in the 2 tubes produced 233 c c. of CO_2 and consumed 275 c c. of O_2 (0°C ., 760 mm.). The close approximation of the respiratory quotient thus obtained with the theoretical oxygen quotient of glycerol should be noted. The relation of the 3 quotients obtained in this experiment may be seen from the following: apparent quotient $= 0.869$; real quotient $= 0.840$; corrected real quotient $= 0.849$.

The relative values of the 3 quotients are subject to change. The real quotient is always lower than the apparent one. The corrected real

TABLE 5

SHOWING CALCULATION OF RESULTS IN DETERMINATION OF CORRECTED REAL RESPIRATORY QUOTIENT FOR *B. SUBTILIS* GROWN ON GLYCEROL AGAR, 2 TUBES, 19 DAYS

Percent	Control	Culture	
CO ₂	0.03	18.17	Apparent gain CO ₂ = 18.14
O ₂	20.93	0.05	Apparent loss O ₂ = 20.88
CO ₂ + O ₂	20.96	18.22	Apparent loss = 2.74
N ₂	79.04	81.78	Calc. apparent manometer = -19.24 mm.
	100.00	100.00	Calc. apparent resp. quot. = 0.869
Corrected analysis*			
CO ₂		17.561	Real gain CO ₂ = 17.531
O ₂		0.048	Real loss O ₂ = 20.882
CO ₂ + O ₂		17.609	Real loss = 3.351
N ₂		79.04	Calculated real manometer = -23.53 mm.
		96.65	Corr. observed manometer = -21.59 mm.
			Calc. real resp. quot. = 0.8395
Volume in jar, c c.....		1700	Bar. at equilibration = 738 mm.
Volume in man. arm.....		3.74	Aqueous tension at 32 C. = 35.674
		1703.74	B - T = 702.326
Volume of 3 tubes.....		27	Calc. real manometer = 23.53
Volume of agar†.....		20	
Volume of water.....		10	B - b - T = 678.796
		57	
Actual air volume, V.....		1646.74	
$V_o = \frac{1 + 0.003665 \ t^{\circ}}{V} \cdot \frac{B-b-T}{760} = 1315.87 \text{ c c. at 0 degrees, 760 mm.}$			
C c at 0 degrees, 760 mm.			
CO ₂ in 2 agar tubes =	2.398		Corr. real resp. quot. = $\frac{233.157}{274.78} = 0.8485$
CO ₂ in water tube =	0.674		
	3.072		
CO ₂ in 2 agar controls =	0.60		Resp. quot. of glycerin = 0.857
CO ₂ dissolved‡	2.472		Percent dissolved CO ₂ = 0.1875
CO ₂ gaseous =	230.685		Tension dissolved CO ₂ = 132 mm.
Total CO ₂ =	233.157		Tension gaseous CO ₂ = 123.1 mm.
O ₂ loss =	274.78		CO ₂ quotient = 0.0107

* Obtained by multiplying the above culture values by the nitrogen factor $\frac{79.04}{81.78} = 0.9665$

† The decrease in the volume of the medium when autoclaving was not determined.

‡ The dissolved CO₂ represents 0.009 in the final respiratory quotient.

TABLE 6

THE RELATIVE VALUES OF RESPIRATORY QUOTIENTS OBTAINED WITH DIFFERENT MEDIUMS

Culture.....	B. subtilis		B. tuberculosis	
	Plain	Glycerol	Glycerol	Glucose
Agar.....				
Quotients				
Apparent.....	0.760	0.864	0.861	0.917
Real.....	0.715	0.833	0.830	0.899
Corr. real.....	0.888	0.848	0.836	0.992

quotient, on the other hand, is higher than the real, for the reason that some additional CO_2 is included in the calculation. If this amount is small, the difference is very slight. But when the organism is making NH_3 or other basic products, the amount of CO_2 which is then taken up may be considerable. In such case, the corrected real quotients may greatly exceed the apparent value. This fact can be readily seen in table 6. The values there given are the average of 4 determinations with each medium in the case of *B. subtilis*; and of 3 such tests with *B. tuberculosis* on each medium.

On reference to table 6, it will be seen that the corrected real respiratory quotient for *B. subtilis* on plain agar is given as 0.888. This value may be taken as correct for the experimental conditions which prevailed; but it probably does not represent the truth as regards the actual intracellular respiratory change. If, as a result of secondary, extracellular reactions, CO_2 is produced, it follows that the total yield of CO_2 will be higher than the amount which strictly is due to cell respiration. Such increase in CO_2 means a corresponding increase in the respiratory quotient.

The secondary change mentioned implies carboxylase action whereby CO_2 and an amine or even NH_3 are formed. A relatively large amount of CO_2 may be thus made within the medium and retained by it because of the simultaneous alkali production.

The effect on the respiratory quotient of the CO_2 thus elaborated will vary with the volume of air contained in the respiratory chamber. If the latter is a test-tube with a capacity of 75 c.c., the quotient for such a container would be much higher than that for a jar of 1,500 c.c. capacity. Actually, the quotient increment due to dissolved CO_2 would in the former be 20 times larger than in the latter.

This can best be illustrated by the examples given in table 7. Under I are given the results of an actual determination made with *B. subtilis*, grown in a tube of plain agar for 120 hours, at 33 C. It will be seen that the corrected real respiratory quotient was found to be 1.002. Since a rubber stopper was used in this experiment, it is certain that the percentage of CO_2 found by analysis was less than it would otherwise be. With no such loss, the quotient would have been appreciably higher than that calculated from the found results, viz., 1.002. This value, though correct, is nevertheless wrong. The true quotient for plain agar should be in the neighborhood of 0.8 rather than of 1.

Now let it be assumed that a like tube of this organism is contained in a large jar the net volume of which, reduced to 0 degrees and 760 mm., is 1,500 c c.; and further, that the percentages found for CO_2 and O_2 are the same as under I. It follows then that the apparent quotient for the jar will be the same as that for the tube. Likewise, the real quotients of the two would be identical. On the other hand, the values for the corrected real respiratory quotients would vary considerably; that for the jar would be 0.724 as against 1.002 for the tube.

TABLE 7
RELATIVE EFFECT ON RESPIRATORY QUOTIENT OF CARBOXYLASE ACTION IN SMALL AND LARGE
AIR VOLUMES

B. SUBTILIS, PLAIN AGAR, 120 HRS. AT 33 C.

	I, Tube	II, Jar
C c. net volume at 0 degrees, 760 mm.	75.56, actual	1500, assumed
Analysis		
CO_2	15.838	
O_2	0.0	
N_2	84.162	
	100.000	
Corrected analysis		
CO_2	14.873	
O_2	0.0	
N_2	79.04	
	93.91	
C c. at 0 degrees, 760 mm.		
Dissolved CO_2	4.629	4.629
Gaseous CO_2	11.215	222.645
Total CO_2	15.844	227.274
O_2 loss.....	15.814	313.950
Quotients		
Apparent respiratory.....	0.755	0.755
Real respiratory.....	0.709	0.709
Corrected real respiratory.....	1.002	0.724
Increase in latter due to dissolved CO_2	0.293	0.015

The correction of the real quotient for the jar would amount to only 0.015, while that for the tube would be 0.293.

This example serves to illustrate the point that the quotient obtained by the jar method approximates closely the true value. The tube method can give good determinations of the quotient, provided carboxylase action is excluded.

HYDROGEN QUOTIENT

When an organism grows as an aerobe, it consumes O_2 and gives off CO_2 , the ratio being expressed by the oxygen or respiratory quotient.

If, on the other hand, the culture is living as an anaerobe, it will obtain its O_2 from the organic compounds in the medium and will give off CO_2 . Usually, in addition, it will produce H_2 unless some reducible substance or acceptor is present. For example, O_2 , if present in less than the inhibitive amount, will be reduced to H_2O or H_2O_2 ; a nitrate to free N_2 and even to NH_3 ; an aldehyde to alcohol, etc.

As a matter of convenience, the $\frac{CO_2}{H_2}$ ratio may be designated as the hydrogen quotient. By filling the culture tube with N_2 instead of with air, and allowing the organism to grow in such atmosphere, positive pressure develops, and analysis reveals the presence of CO_2 and H_2 . Thus, taking from our records, at random, a culture of *B. aerogenes* (Jordan), grown on 1% glucose, it developed in $11\frac{1}{2}$ hours a corrected pressure of +205.6 mm.; barometer, 743; temperature, 32.8 C. $B - T = 706$. Analysis gave the values found under I, while the recalculated or real figures, on the nitrogen basis, are under II. The composition of the air in the tube, at the start is given under III.

	I	II	III
CO_2	13.98	18.299	0.0
H_2	9.86	12.906	0.0
O_2	0.0	0.0	0.31
N_2	76.16	99.69	99.69
	<hr/> 100.00	<hr/> 130.895	<hr/> 100.00

Since the H_2 as well as CO_2 is made by the organism, it follows that multiplying the respective analytic values by the correction factor deduced below in no wise alters the ratio. Hence, there is no distinction between the apparent and the real quotient as is the case with the respiratory quotient. The hydrogen quotient $= \frac{13.98}{9.86} = 1.418$. However, the correct quotient requires the inclusion of the CO_2 dissolved in the medium, in which case the value would become larger than that given.

When an initial, small amount of O_2 is present in an anaerobe tube, it may disappear, thus apparently indicating utilization by the organism. It is more probable, however, that a secondary reaction is involved in which water is formed at the expense of the H_2 . Consequently, the H_2 quotient would be higher than otherwise would be the case.

Assuming that no N_2 is made, the sum of CO_2 and H_2 , or 23.84, is the apparent gain. This multiplied into $(B - T)$ or 706 = 168.31 as the calculated apparent manometric reading, which is appreciably different from the corrected observed value of 205.6.

It is to be noted, however, that the N_2 which at the outset was 99.69, in the foregoing analysis is represented by 76.16%. Hence, proceeding as before

$$\frac{N_2 \text{ of control}}{N_2 \text{ of culture}} = \frac{99.69}{76.16} = 1.30895,$$

which is the nitrogen or correction factor. The real gain is 30.89%, which multiplied into $(B - T)$ or 706 = 218.08 as the calculated real pressure, which is only 12.48 mm. higher than the corrected observed figure.

CALCULATIONS BASED ON MANOMETRIC READINGS

The observed manometric reading, when the aqueous tension is fully provided for, and, when corrected as heretofore given, represents the real loss or gain in pressure brought about by the growth of the culture. It is possible to develop formulas which will indicate the several factors involved: thus, R. Q. = respiratory quotient, B = barometer at equilibration, P = corrected manometric pressure, T = tension at t° at last reading, L = real loss in per cent., G = real gain in per cent., x = real O_2 loss in per cent., y = CO_2 dissolved in the medium, and calculated as percentage of the final volume at 0 degrees 760 mm.

The observed loss in gas volume, indicated by a negative pressure, is chiefly due to the oxidation reaction, but in addition it is somewhat increased by the solution of a portion of the CO_2 , which is produced, in the medium, water, rubber stopper or connection. Since, as will be shown in tables 9 and 10, Part II, the negative pressure in a tube closed with a rubber stopper may be 10 or 20 mm. higher than one with paraffin, sealing-wax or glass seal, it follows that 1.5-3 or more per cent. of CO_2 is thus removed corresponding to the partial pressure of the CO_2 absorbed by the rubber. Hence, the rubber stopper should be avoided when exact manometric observations, and exact analyses are to be made. When this is done, the factor y refers to the CO_2 dissolved in the medium.

The resulting pressure, or P, may be expressed by equation (1)

$$(1) \quad P = (B - T) \cdot \frac{L}{100}$$

But considering the factors involved, that is, the respiratory quotient, the O_2 consumed and the dissolved CO_2 , we arrive at equation (2)

$$(2) \quad P = (1 - R.Q.) \cdot (B - T) \cdot \frac{x}{100} + (B - T) \cdot \frac{y}{100}$$

which may be written in the form (3)

$$(3) \quad \frac{P \cdot 100}{B - T} = (1 - R.Q.) \cdot x + y$$

On combining (1) and (2) we have (4):

$$(4) \quad (B-T) \cdot \frac{L}{100} = (1 - R.Q.) \cdot (B-T) \cdot \frac{x}{100} + (B-T) \cdot \frac{y}{100}$$

and on cancelling this becomes (5):

$$(5) \quad L = (1 - R.Q.) \cdot x + y$$

The loss may also be derived from (1), or by combining (3) and (5) and we have (6):

$$(6) \quad L = \frac{P \cdot 100}{B-T}$$

The foregoing formulas apply to a negative pressure. In the case of a positive pressure, the term $(1 - R.Q.)$ becomes $(R.Q. - 1)$; and the $+$ sign before y changes to $-$.

The observed manometric reading is an excellent check on the correctness of the analysis provided proper equilibration is made at the start of the experiment. The corrected observed reading should agree within 1 or 2 mm. with the calculated pressure, but with agar mediums this is not usually the case (table 8). The pressure, when negative, is calculated from equation (1), in which L , the real loss, is determined as shown in table 4 or 5. It may be arrived at directly from the formula

$$(7) \quad L = 100 - \frac{100 \cdot N_2 \text{ in control}}{N_2 \text{ in culture}}$$

The apparent loss must not be used in calculating the manometric reading. The pressure, when positive, is calculated from equation (9).

The calculation of the manometric readings for anaerobes, grown in N_2 has been referred to above, under hydrogen quotient. On the assumption there given, the real gain, or G , is expressed by the formula

$$(8) \quad G = \frac{100 \cdot N_2 \text{ in control}}{N_2 \text{ in culture}} - 100$$

And

$$(9) \quad P = (B - T) \cdot \frac{G}{100}$$

The calculation for cultures grown in pure oxygen is necessarily somewhat different than when N_2 is present. In such case, the manometric reading must be made use of to obtain the desired factor. Thus, assuming that the observed manometric reading is -49 , that 100% CO_2 was found, and that $B - T$ is 700, formula (6) becomes:

$$L = \frac{49 \cdot 100}{700} = 7\%$$

And $\frac{100 - 7}{100} = 0.93$, the desired factor.

Hence, $100\% CO_2 \times 0.93 = 93$ as the real % of CO_2 made.

And the real resp. quotient $= \frac{93 \text{ per cent. } CO_2}{100 \text{ per cent. } O_2} = 0.93$

The formulas render it possible to calculate any one of the terms provided the others are known. The degree of accuracy will, of course, depend on the correctness of the known terms.

By way of an example we may apply formula (3) to determine the value of y , the percentage of dissolved CO_2 , using the data given in table 5. The formula becomes:

$$\frac{23.53 \times 100}{702.326} = (0.1515) \times 20.882 + y$$

And y is found to be 0.187%, which is the same value as that in the table. If a rubber stopper is used to seal the tube, the value of y will indicate the amount of CO_2 dissolved by the stopper and the medium.

Another example of the usefulness of the manometer is shown by the possibility of making a series of calculations when the analytical data are incomplete. Thus, in an experiment with an *h*-tube which had, in the side-arm, broth inoculated with *V. septique* and, in the main arm, raw sterile potato, 27.75% of CO₂ was found while the oxygen of the air was completely removed, thus indicating a real O₂ loss of 20.93%. The real gain, calculated on the usual N₂ basis, was 9.398%, which multiplied into 706, or B — T, gave +66.3 as the calculated manometric reading. Since the corrected observed manometer was +148.82 (P), it was evident that some other gas, probably H₂, was present. The H₂ had not been tested for, and it was desirable to ascertain the probable amount present.

Assuming that the observed figures are practically correct, it becomes possible to calculate in this example:

- | | |
|--|--|
| (1) Percentage of real gain | (5) Percentage of apparent H ₂ and N ₂ |
| (2) The N ₂ factor | (6) Respiratory quotient |
| (3) Percentage of real CO ₂ | (7) Hydrogen quotient |
| (4) Percentage of real H ₂ and N ₂ | |

According to formula (6):

$$\text{Real gain} = \frac{P \times 100}{B - T}, \text{ and hence}$$

$$(1) \text{ Real gain} = \frac{148.82 \times 100}{706} = 21.08\%$$

This gain is over and above the original 100%
Hence the total percentage = 121.08

- | | |
|--|--------------|
| (2) Nitrogen factor = 1.2108 | |
| (3) Analysis gave 27.75% CO ₂ which multiplied by N ₂ ^f | = 33.60 real |
| Likewise, the N ₂ ^f × the undetermined N ₂ would | = 79.04 real |

(4) The difference, 121.08 — 112.64 = hydrogen	112.64
	8.44 real
	<hr/> 121.08

Since the percentage of a gas found when multiplied by the N₂ factor gives the real percentage, it follows from (4) and (2)

$$(5) \text{ That the apparent percentage H}_2 = \frac{8.44}{1.2108} = 6.97$$

To arrive at the respiratory quotient, we have

$$(6) \frac{\text{CO}_2 \ 33.60 - 0.03}{\text{O}_2 \ 20.93 - 0} = \frac{33.569}{20.93} = 1.604$$

And similarly the hydrogen quotient is obtained from

$$(7) \frac{\text{CO}_2 \ 33.569}{\text{H}_2 \ 8.44} = 3.977$$

The high values obtained in (6) and (7) merely show that anaerobic respiration followed the removal of O₂.

By way of a summary, the results arrived at above may be arranged in a tabular form. The apparent values are given under I; the real values, under II; while the composition of the air in the tube, at the start, is given under III.

	I	II	III
CO ₂	27.75	33.60	0.03
O ₂	0.0	0.0	20.93
N ₂	65.28	79.04	79.04
H ₂	6.97	8.44	0.0
	<hr/> 100.00	<hr/> 121.08	<hr/> 100.00

By way of a check on the foregoing method of calculation, the analytic data obtained with *B. aerogenes* were used to find the amount of H₂, assuming that it had not been determined. The value for H₂ thus obtained was 8.81, while actually 9.86 had been found. The difference was due to the fact that the corrected observed manometric reading was 12.48 mm. lower than the calculated reading. The calculation based on the latter reading gave 9.86, which was the amount found by analysis. (See under Hydrogen Quotient.)

Paradoxical though it may seem, the observed manometric readings for a given organism and medium may be found to differ considerably. A little consideration will show that, apart from the initial barometric pressure, this variation depends on the volume of the air in the container and on the volume of the medium. If these volumes are kept constant, the final manometric readings likewise should be constant. Differences are likely to be noted, due perhaps to uneven inoculation or multiplication. On the other hand, if the volume of the medium is constant (10 c.c.) but the air volume varies, e. g., from 50 c.c. in a culture tube to 2,000 c.c. in a culture jar, the final manometric readings, after all of the O_2 has been absorbed, will show a marked difference. This may be seen clearly from the following examples in which the corrected, real manometric values are deduced for a culture grown (1) in an anaerobic jar, and (2) in an ordinary test-tube. The reading of the manometer of the latter is just about double that of the former.

If we conceive of the complete oxidation of glycerol in a closed vessel with all of the O_2 in the air being consumed, the respiratory quotient will be 0.857, and the percentage of CO_2 produced can be arrived at from the formula

$$\begin{aligned} \text{Resp. quot.} &= \frac{CO_2 \text{ produced}}{O_2 \text{ lost}} \\ 0.857 &= \frac{CO_2 \text{ produced}}{20.93} \end{aligned}$$

On subtracting the CO_2 produced, or 17.937, from 20.93, we have in this case the real loss, or 2.993%. By multiplying 702.326, the assumed corrected barometric pressure at 32 C. (B — T) by this value, we obtain the real manometric reading, or —21.02 (equation 1).

It will be seen that the CO_2 produced = 17.937%. This, then, would be the value which one could expect to find on analysis, provided no absorption of CO_2 by water or other medium took place.

However, the culture tube does contain, for example, 10 c.c. of agar medium, and this will absorb a definite amount of CO_2 , which analysis may show to be 1.25 c.c. at 0 degrees and 760 mm. pressure.

If we now assume the gas volume, reduced to 0 degrees, and 760 mm. to be 1320.08 c.c., and to this volume add the c.c. of dissolved CO_2 , we obtain the total gas. The percentage of the gas dissolved is now obtained.

$$1321.33 : 1.25 : : 100 : \times = 0.0948\%$$

On multiplying by this percentage, the corrected barometric pressure at 32 C. (B — T), one arrives at the pressure decrease due to removal of CO_2 by solution.

$$\begin{aligned} 702.326 \times 0.0948 &= \frac{0.666}{21.02} \text{ real manometer} \\ &= -21.686 \text{ corrected real manometer (equation 2)} \end{aligned}$$

Again, if we assume that the reduced volume of the gas in a culture tube is 40 c.c., and adding to this the 1.25 c.c. of CO_2 dissolved by the medium, we have a total gas of 41.25 c.c.

$$41.25 : 1.25 : : 100 : \times = 3.03\%$$

$$\begin{aligned} \text{And } 702.326 \times 3.03 &= \frac{-21.28 \text{ mm. pressure due to solution}}{21.02 \text{ real manometer}} \\ &= -42.30 \text{ corrected real manometer.} \end{aligned}$$

It will be seen from the foregoing example that, with complete consumption of O_2 , the large jar would show a negative pressure of —21.6, whereas the culture tube would indicate —42.3. As a matter of fact, even greater differences than here shown have been observed.

Another example which seemingly calls for an explanation concerns the experiment given in table 4. The corrected observed pressure in that case was —162 mm., whereas ordinarily the tubercle bacillus, when growing in air, produces a pressure of about —32 mm. On reference to formula 2, it will be seen that the pressure depends largely on the value of x , the percentage of O_2 consumed. Obviously, with an atmosphere of 100% O_2 , the pressure should be about 5 times that which would be obtained with ordinary air. Further, the factor y would be represented by the partial tension of the CO_2 dissolved from an atmosphere containing 86% of CO_2 and having a volume of about 40 c.c. It would therefore be at least 5 times that which would be obtained from one which has only 17% CO_2 . The result is that the manometric pressure observed in this experiment is about 5 times as high as that of a like culture grown in air.

Aqueous Tension.—Working with slanted agar, notwithstanding the greatest care in equilibrating the manometer at the start, the observed manometric reading was found to be almost invariably ± 10 mm. below the calculated value. This depression might be assumed to be due to the production of N_2 or other gas by the organism, or to a rise in aqueous tension due to a slow saturation of the air in the culture tube. Considerable effort was devoted to ascertain the reason for this discrepancy. Eventually, the latter factor was found to be the sole cause of the observed depression.

When the manometer was equilibrated after having been kept for 2 hours in the hot-room, it was assumed that the aqueous tension had been fully established. The agar medium, however, is distinctly hydrophilic, and the result is that the equilibration is made against the unsaturated air in the culture tube. With the development of the culture on the medium, moisture is brought to the surface, and the aqueous tension is increased. The manometric reading is consequently depressed by the added vapor tension.

It may be added that the discrepancy is greater with a 2% agar medium than with one that has 1%. With the former, the calculated manometric reading was usually 15 mm. higher than the observed reading, while with the latter the difference was about 10 mm. The results with liquid mediums show close agreement of the calculated and observed readings.

The point brought out may be illustrated by an experiment with *B. subtilis*. Each of 4 bulb tubes provided with a side-arm and having a capacity of about 150 c.c., received 10 c.c. of the nutrient medium. The latter contained 1% agar and 5 % glycerol. After sterilization at 120 C. for 20 minutes, the tubes were slanted over night. The next morning, 0.5 c.c. of sterile distilled water was placed in the bulb of each of 2 of the tubes, which were then placed in the hot-room. The water in the bulbs was gently heated several times to cause an abundant condensation on the walls of the tubes. The 4 tubes were then inoculated with the culture and closed with sealing-wax. Each tube was then attached by means of the side-arm to a manometer which was equilibrated an hour later. The tubes were analyzed at the end of 49 hours. The results of this experiment are given in table 8.

It will be seen on reference to table 8 that the 2 control tubes had 9 mm. less pressure than the 2 tubes which had a little water in the bulb and had been steamed to saturate the contained air. The comparison of the calculated real manometric values with the corrected observed readings reveals as close a concordance as may be expected (± 1 mm.)

for tubes 1 and 2. The difference for tubes 3 and 4 is 11.1 and 9.4 mm., respectively.

The foregoing experiment shows conclusively the error of assuming that the agar provides the full aqueous tension at the time of equilibration. As a hydrophilic substance it tends to reduce the amount of water vapor present. It is possible that the dry cotton plug may also absorb some of the vapor and thus lessen the initial aqueous tension.

TABLE 8
INFLUENCE OF AQUEOUS TENSION UPON THE MANOMETRIC READINGS
B. SUBTILIS ON GLYCERINE AGAR, 49 HRS. 32°, C.

Bulb with.....		Water		No Water	
Tube.....		1	2	3	4
Hrs.					
Equilibrated	0.....	0	0	0	0
	1.....	0	0	+2	+2
	2.....	-1	-1	2	2
	5.....	2	3	+1	+1
	11.....	10	10	-5	-1
	25.....	18	19	12	9
	37.....	22	22	14	13
	49.....	-24	-24	-15	-15
Corrected manometer.....		24.38	24.38	15.15	15.21
Calculated manometer.....		24.03	25.32	26.26	24.61
Difference.....		-0.35	+0.94	+11.11	+9.40
Analyses					
CO ₂		18.01	17.80	17.77	17.22
O ₂		0.22	0.28	0.20	0.94
N ₂		81.77	81.92	82.03	81.84
		100.00	100.00	100.00	100.00
Corrected analyses					
CO ₂		17.41	17.17	17.12	16.63
O ₂		0.21	0.27	0.19	0.91
N ₂		79.04	79.04	79.04	79.04
		96.66	96.48	96.35	96.58
Real respiratory quotient.....		0.839	0.830	0.824	0.829

The difficulty can be overcome by introducing into the tube an excess of moisture. The method used in the foregoing experiment is not applicable to the ordinary tube. The same result, however, can be obtained by the injection of a little steam into the cotton plug or through the side-arm, if the tube has one. For this purpose an Erlenmeyer flask, containing some water, is provided with a stopper, into which is inserted a bent tube. This is connected by a short piece of rubber tubing to a finely drawn out glass tip. On gently heating the water, a fine jet of steam may be produced. By means of a finely drawn out glass rod

(4 mm.) which is rolled back and forth, a channel can be readily made in the solid cotton plug. On inserting the steaming tip into this channel, the inner wall of the tube can be quickly covered with condensed moisture.

As mentioned before, the manometric reading is an excellent check on the analysis. With care to provide the full aqueous tension at the time of equilibration, the calculated reading should agree closely with the observed reading. When such agreement does not exist, it shows that an error is present, which may be in the analysis of the control or of the culture. Apart from this, the error may be due to formation of a gas, such as H_2 , N_2 , etc., by the culture. The manometer, therefore, may be found to be a serviceable indicator of the production of such gases or of products which have high tensions.

SUMMARY

A compensation manometer is described which permits observation of the pressure changes taking place in a culture.

The study of cultures in different gases, or in varying mixtures of gases, is made practicable.

A manometric method of analysis of the gas exchange due to organisms is developed.

The buret method of analysis is described in detail, with procedures for sampling the gas content.

An accurate process is given for the determination of the CO_2 taken up by the culture medium.

The correct interpretation of analytic values is considered with its bearing on the true respiratory or oxygen quotient and on the true hydrogen quotient.

MICROBIC RESPIRATION

II. RESPIRATION OF THE TUBERCLE BACILLUS

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SYNOPSIS

Introduction

Methods

Respiratory Quotient

Glycerol; Glucose; Plain Agar; Serum Agar

Oxygen Requirement

Required Air Volume; Tubes Sealed in Flame; with Sealing-Wax; Paraffin; Rubber Stoppers

Manometric Changes

Aerated and Un aerated; Analyses; Growth; Tubes Sealed in Flame; with Sealing-Wax; Paraffin; Rubber Stoppers

Increased Oxygen Tension

Growth in 30, 40, 60, 80 and 100% Oxygen

Decreased Oxygen Tension

Growth in 10, 5, 3, 1 and 0.5% Oxygen

Increased Carbon Dioxide Tension

Growth in 30, 40, 50, 60, 80 and 90% CO₂

Decreased Carbon Dioxide Tension

Growth in Presence of Alkali; Desiccation

Moisture Requirement

Mercury Tension

Inhibition of Growth; Black Cultures

Viability and Virulence

Summary

The study of the respiration of the tubercle bacillus is of considerable interest. Exact knowledge as to the amount and rate of gas change has been lacking, and it is precisely because of this that a careful investigation was needed in order to have data which would permit a true interpretation of hitherto published results, and, at the same time, supply a background for further studies of this important organism. It will be shown that the respiratory changes of the tubercle bacillus are quantitatively the same as those of other organisms, although the rate of exchange is necessarily slower.

Respiration is a fundamental phenomenon of life; it is the one sure sign of intracellular activity; and its absence or cessation is an unerring indication of impaired vitality and death. In the case of a slow-

growing organism, such as the tubercle bacillus, it is not possible to assert that a subculture is alive and multiplying until the mass has perceptibly increased, which occurs usually after the lapse of a week or two. By the manometric method, the viability of the transplant is indicated within a few hours, provided the medium is such as to give a respiratory quotient which is appreciably less than 1. When the indications of the manometer are supplemented by exact analyses of the gaseous content of the culture tube or jar, it becomes possible to understand the behavior of this organism under varied conditions.

As the tubercle bacillus is usually grown in tubes on slanted solid mediums, it was desirable at the outset to ascertain the changes which took place under those conditions. The basic facts thus obtained become applicable to other mediums regardless of the kind of container. Although the tubercle bacillus is generally recognized to be an obligative aerobe, it is nevertheless true that nothing definite was known as to the amount of O_2 which it required for growth, and that even less was known as to its response to maximal and minimal tensions of O_2 or of CO_2 .

To prevent desiccation, it is the common practice to seal the culture tube in some way. Sealing-wax, paraffined cotton stoppers, rubber caps, rubber stoppers, and corks are usually employed for this purpose on the assumption that there is enough oxygen in the tube, and that any additional, needed oxygen can readily enter through such seals. It will be shown that the former assumption is incorrect, while the latter rests purely on the element of chance which at times may be favorable, at other times unfavorable. Consequently, the results range from no growth to a rich one.

Another factor which may be mentioned in this connection is that of moisture. Only too often solid mediums are used without due regard to the moisture requirements of the organism. The result is a somewhat dry, restricted growth quite unlike that which is obtainable under really moist conditions.

METHODS

The compensation manometer has been found to be of greatest help in the study of the gas changes going on within the culture tube, especially so with glycerol agar as the medium. As stated, if the organism is alive, it reveals that fact by a negative pressure which rises fairly rapidly to a maximum where it is then maintained for weeks, the duration of the experiment, provided no leakage or loss develops.

Before taking up the experimental work, it will be well to give in some detail the general procedure followed.

A human strain of the tubercle bacillus, the well-known H 37, was kindly supplied by Major P. A. Schule of the Army Medical School and was used throughout this study. Real basic facts can be obtained only by an intensive study of a single strain, and for that reason the inclusion of a variety of human strains was deliberately avoided.

The culture tubes were usually 20 x 200 mm., and were of different form according to the purpose in view. Straight tubes, each having a constricted side-arm (fig. 2 C, Part I), were found to be especially useful in the study of the different methods of sealing tubes. The side-arm was always closed with a loose pledget of cotton. The average full capacity of the empty tube was 56 c.c.

The cotton plugs were rolled as firmly as possible. If this was not well attended to, those tubes which were covered with sealing-wax or paraffin were likely to leak when a negative pressure was developed by the growth; especially when this pressure was further increased at the time of the withdrawal of a sample of the air for analysis. When tubes were placed in jars for the purpose of exposing the organism to a given atmosphere, it was essential to promote the diffusion of gases by using cotton plugs, which were made as loose as possible.

A stock of 1% agar, sufficient for several months' work, was prepared. Selected chopped beef was digested in the icebox, over night, with 2 parts of distilled water.

To the meat extract thus prepared, 1% of Witte's peptone and 0.5% sodium chloride (K) were added; the liquid was then carefully adjusted to P_H 7.4: boiled, filtered and 1% agar added.

The agar medium was then filled into a number of 125 c.c. sterile Erlenmeyer flasks, and autoclaved at 110 degrees for 20 minutes. For a given experiment, one or two of the flasks were liquefied by heat and 5% of Kahlbaum's glycerol was added. The medium was measured by means of a standard pipet into sterilized tubes which were then autoclaved at 110 degrees for 20 minutes; after this the tubes were slanted so that the agar surface was about 100 mm. in length.

On account of the softness of the medium it was not inoculated, as a rule, until after it had been allowed to set for from 12 to 24 hours.

The inoculations were made by means of a spatula (Roux type), 25 cm. long. This was made of a No. 9 gage iron wire, the end being hammered out to a thin blade of 4 x 5 mm. After being sterilized in the flame, the spatula was allowed to cool for 3 minutes before it was used. A heavy transfer of the culture was always made.

In much of the early work in this study, the inoculated tubes were closed either with sealing-wax, paraffin, rubber stoppers, or sealed in the flame. When the tube which was closed with sealing-wax intended to be used with a manometer, a Hg seal cup (fig. 12, Part I) was attached to the end. Before proceeding to draw a sample of air from the tube for analysis, this cup was filled with Hg. In some tests, as, for example, tubes 7-9 in table 6, the Hg seal was employed from the start. When a glass seal was to be made, the cotton plug was removed from the tube, which was then closed in the blast lamp. In the tests in which a rubber stopper seal was desired, No. 3 or No. 4 stoppers were used after having been cleaned and treated with glycerol as described later. The closure with paraffin will also be discussed later.

The connection of the side-arm of the culture tube with the manometer is a matter of importance. A rubber tubing could not be found to answer

this purpose. An entirely satisfactory connector was made out of a solid rubber stopper (No. 25 A. H. T.) 38 mm. long, the ends showing diameters of 28 and 34 mm., respectively. A smooth 4 mm. hole was cut through the stopper by means of an electric drill which was kept moistened with a mixture of sodium hydrate and glycerol. This perforated rubber stopper, when used with glass to glass connections made a gas-tight joint.

Before use, all rubber stoppers were first soaked in 20% KOH for several days, then boiled; the excess of alkali was removed by repeated washing in distilled water, after which they were boiled with 4% HCl and allowed to remain in the acid for several days. After being rinsed several times with distilled water and boiled, they were put in glycerol and autoclaved at 110 C. for 30 minutes. The stoppers remained in glycerol until needed. A dry rubber stopper cannot be used in a test-tube or bottle because of inevitable leakage. But when the stopper is cleaned and soaked in glycerol in the manner given, it will hold a negative pressure of 200 mm. for days and weeks.

Before attaching the perforated stopper to a manometer, its bore was probed so as to remove any excess glycerol. In order to insure a perfectly tight connection it was found advisable to apply a screw hose clamp ($\frac{3}{4}$ inch) at each end of the stopper. This was then attached to the end of the manometer, and finally the side-arm of the culture tube was inserted so as to have a glass to glass connection.

In a limited number of tests, the side-arm of the culture tube was fused on to the end of the manometer. Such tests served as checks on the rubber stopper connectors. The manometers, with the attached tubes, after closing cocks 1 and 3, were placed in the hot-room at 37 C. Three hours later, after they had taken on the temperature of the room, the manometers were equilibrated, and cocks 1 and 3 were again closed.

The Novy jars, of different capacities, were found to be particularly useful in the determination of quotients, and in the study of the effect of varying concentrations of different gases. In special cases, they were supplemented by bottles having a capacity of 8 to 20 liters. The details as to the use of these jars and bottles will be given later.

RESPIRATORY QUOTIENT

The methods for the determination of the respiratory quotient have been given in Part I. The culture tube can be used for this purpose, but to obtain the utmost accuracy it is necessary to use the anaerobe jar. Incidentally, because of the large volume of air present, the jar method supplies quantitative data which can only be approximated by serially aerated culture tubes. That a definite and very large amount of oxygen must be provided in order to secure a good growth of the tubercle bacillus in a culture tube, can be established readily by the jar method.

Glycerol Agar.—The loosely plugged sterile culture tubes (20 x 150 mm.) received 10 c.c. of the nutrient medium which contained 1% agar and 5% glycerol. They were autoclaved at 120 C. for 20 minutes, slanted over night, and then were inoculated, by means of the spatula, with rich young cultures. One or two of the tubes were placed in an anaerobe jar together with an open tube containing 10 c.c. of distilled water. About 5 drops of water were

placed on the bottom of the jar to provide quickly full aqueous tension. The jars were then sealed, attached to manometers, and equilibrated in the manner described in Part I (fig. 6). They were kept at 37 C. for 26-27 days, when the gas content of each was analyzed. The results of 3 experiments of this kind are given in table 1.

TABLE 1
ANALYSES AND QUOTIENTS OF B. TUBERCULOSIS GROWN ON GLYCEROL AGAR

Experiment No.	1	2	3
No. of days.....	27	27	26
No. of tubes.....	1	1	2
Net air volume.....	1699.38	1810.35	1786.5
Barometer.....	741	741	744
Temperature, C.	37.7	37.7	37.3
Corr. obs. man.	-13.17	-8.18	-22.50
Calc. real man.	-11.15	-12.75	-22.25
Analyses			
CO ₂	8.967	8.830	15.244
O ₂	10.699	10.647	3.106
N ₂	80.334	80.523	81.650
	100.000	100.000	100.000
Corr. analyses*			
CO ₂	8.822	8.667	14.757
O ₂	10.527	10.451	3.007
N ₂	79.040	79.04	79.039
	98.389	98.158	96.803
C c. at 0 C., 760 mm.			
Dissolved CO ₂	0.642	0.776	2.07
Gaseous CO ₂	117.609	122.800	205.23
Total.....	118.251	123.576	207.30
O ₂ loss.....	139.159	148.989	249.77
Quotients	Average		
Apparent resp.	0.8609	0.8735	0.8558
Real resp.	0.8303	0.8451	0.8242
Corr. real resp.	0.8364	0.8497	0.8294
CO ₂	0.0073	0.0055	0.0063
			0.8535
			0.8217
			0.830
			0.0101

* The initial gas content was assumed to be that of pure air, viz.: CO₂, 0.03; O₂, 20.93; and N₂, 79.04.

It is to be noted that the observed negative pressure (-22) with 2 tubes in the jar (exper. 3) was about twice that obtained with single tubes. As might be expected, the O₂ consumption and CO₂ production was most marked in the jar which contained 2 tubes. It has been shown in Part I that the pressure observed depends chiefly on the amount of O₂ consumed. The O₂, however, was not completely exhausted in the time allowed for the test. The CO₂ production in this experiment amounted to 15.2%. Had all of the O₂ been consumed, the yield of CO₂ would have exceeded 17.5%, and the negative pressure would have been still further increased. The growth in all of the tubes was very rich.

Of particular interest are the values obtained after reduction of the gases to standard conditions, 0 degrees and 760 mm. It will be seen from the table that per tube the CO₂ production was 118, 123 and 104 c.c., while the O₂ loss was 139, 149 and 125 c.c., respectively. The further discussion of these values will be taken up in connection with table 5.

The values for the 3 kinds of respiratory quotients, resulting from growth on glycerol agar, are given in table 1. For a full discussion of the respiratory quotient, the reader is referred to Part I (table 6). It will be seen that the apparent quotient was higher than the real and that the corrected real quotient was but slightly higher than the latter. This was due to the fact that the amount of CO₂ taken up by the medium and the water in the jar was slight. In fact, the 10 c.c. of agar took up only a little more than did the 10 c.c. of water, showing that, on glycerol agar practically no alkali was made by the tubercle bacillus.

In this respect, the behavior of the growth on glycerol agar was different from that on glucose agar. On the latter, the organism produced a notable amount of alkali, as indicated by the amount of CO₂ taken up, and hence such cultures gave a corrected real respiratory quotient which exceeded the apparent quotient. A comparison of the relative amounts of CO₂ taken up by glycerol and glucose agar will be found in table 3.

The corrected real quotients ranged from 0.83 to 0.85, the average of 3 separate determinations being 0.836. Hence the quotient found closely approximates the theoretical value for glycerol, which is 0.857. To illustrate the extent of the divergence, it may be stated, assuming 100 c.c. of CO₂ to be produced, that 119.6 c.c. of O₂ would have to be consumed in order to give a respiratory quotient of 0.836; while 116.7 c.c. would be needed for the theoretical quotient 0.857. The difference of 2.9 c.c. would represent only 0.2% if the gas volume were 1,500 c.c.

Glucose Agar.—This nutrient medium contained 1% agar and 2% glucose. The arrangement of the experiments was otherwise the same as those with glycerol agar. The tubercle bacillus grew fairly well on glucose agar, but the growth in 4 weeks was not as rich as that on the glycerol medium.

The 3 experiments given in table 2 were started at the same time. In exper. 1, the gas sample, drawn on the 26th day, showed only a slight change in the composition of the air. Accordingly, the other 2 experiments were allowed to continue for 85 days. Even at the end

of that long period, there were still 10.5% of O_2 in each jar. Exper. 2 and 3 in table 2 may well be compared with exper. 3 in table 1. In the latter, in 26 days, the O_2 content was reduced to 3.1%. Hence it is clear that the tubercle bacillus on glucose agar did not grow as fast or respire as well as it did on glycerol agar.

TABLE 2
ANALYSES AND QUOTIENTS OF *B. TUBERCULOSIS* GROWN ON GLUCOSE AGAR

Experiment No.	1	2	3
No. of days.....	26	85	85
No. of tubes.....	2	2	2
Net air volume.....	1614.35	1507.8	1658.07
Barometer.....	748	748	748
Temperature, C.	37.2	37.1	37.1
Corr. obs. man.	-3.04	-1.03	-4.08
Calc. real man.	-2.84	-5.84	-8.98
Analyses			
CO ₂	3.514	9.650	9.346
O ₂	17.095	10.646	10.588
N ₂	79.361	79.704	80.066
	100.000	100.000	100.000
Corr. analyses*			
CO ₂	3.529	9.570	9.226
O ₂	17.026	10.557	10.452
N ₂	79.04	79.04	79.04
	99.595	99.167	98.718
C c. at 0 C., 760 mm.			
Dissolved CO ₂	6.612	10.039	10.162
Gaseous CO ₂	45.623	115.760	122.158
Total.....	52.235	125.799	132.320
O ₂ loss.....	50.904	125.868	139.188
Quotients	Average		
Apparent resp.	0.917	0.916	0.935
Real resp.	0.899	0.896	0.923
Corr. real resp.	0.902	1.026	0.999
CO ₂	0.105	0.145	0.087
			0.901
			0.878
			0.951
			0.083

* See footnote to table 1.

An examination of table 2, especially of the data for exper. 1, after reduction to 0 degrees and 760 mm., will show that the O_2 consumed was only about one fifth of that used up in the same time in exper. 3, table 1, while the yield of CO_2 was only about one fourth. The O_2 loss in the latter, in 26 days, was 250 c c., while, in the same time, in exper. 1, table 2, it was only 50 c c. Even at the end of 85 days, the O_2 loss was only about one-half of that consumed on glycerol agar in 26 days. It follows, as stated above, that the gas exchange of the tubercle bacillus when grown on glucose agar was not as pronounced as it was on the glycerol medium.

The low gas exchange obtained with cultures grown on glucose agar cannot be ascribed off-hand to poorness of growth. While the cultures were not as rich as those on the glycerol medium, they were nevertheless good. It would have been of interest to know the relative weights of the dried cultures. It would then be possible to draw definite conclusions as to the reason for the low respiratory change on glucose agar. There is the possibility that, in part at least, the glucose molecule is assimilated to form cell matter. In this way, the cell might receive a needed supply of combined oxygen without producing the large amounts of CO_2 which are given off on glycerol mediums. On the other hand, it is equally conceivable that glucose is not as readily oxidized by the organism as is glycerol, and hence it does not furnish growth energy to the same extent as does the latter.

TABLE 3

PARTITION OF DISSOLVED CO_2 , C C., AT 0 C., 760 MM., PER 10 C C. OF AGAR AND WATER

Agar.....	Glycerol			Glucose			Serum		
Experiment No.	1	2	3	1	2	3	1	2	3
No. of days.....	27	27	26	26	85	85	28	28	28
In 10 c c. agar.....	0.38	0.49	0.73	3.27	4.75	4.88	2.83	3.01	3.45
In 10 c c. water.....	0.26	0.28	0.60	0.14	1.06	0.80	1.36	1.44	1.40
C c. gaseous CO_2 per tube.....	117.61	122.80	102.11	22.81	57.88	61.08	13.98	16.95	16.80
Percent. gaseous CO_2 in jar.....	8.97	8.83	15.24	3.54	9.57	9.23	1.94	2.12	2.60
CO_2 quotient.....	0.0055	0.0063	0.0101	0.145	0.087	0.083	0.227	0.199	0.226

Another striking fact, revealed by the analyses, was the large amount of CO_2 taken up by the medium. The values for dissolved CO_2 , as given in tables 1 and 2, include the amount taken up by 10 c c. of water. The amount taken up by 10 c c. of the glucose medium was more than 10 times that taken up by a like volume of water in the glycerol experiments. The actual volume of dissolved CO_2 at 0 degrees and 760 mm. per tube of 10 c c. of glucose agar, compared with that contained in glycerol agar is given in table 3.

It will be seen that the glycerol agar took up only a little more CO_2 than did the same volume of water. This indicated that mere solution took place and that the tubercle bacillus in growing on glycerol agar did not liberate an appreciable amount of NH_3 or other base.

On the other hand, the glucose agar medium dissolved 10 times or more CO_2 than did the same volume of water. Clearly, this large volume of gas was not in mere solution. It follows that the major part of it was in a combined state.

It might be assumed that this fixation of CO_2 was due to the presence of impurities in the glucose, but such was not the case. In a control experiment made to test this point, 2 tubes each of glucose and of glycerol agar were placed in a jar, the air of which contained 11.5% of CO_2 . After 2 days, at 37 C., the dissolved CO_2 in these tubes was determined in the usual way. The result, expressed in c.c. at 0 degrees and 760 mm. was as follows:

	Tube 1	Tube 2
Glucose agar.....	1.47	1.48
Glycerol agar.....	1.52	1.59

It is evident, therefore, that the glucose agar itself contained nothing which would account for the large amount of CO_2 which was found. Hence the conclusion is justified that basic products are formed by the tubercle bacillus when it is grown on glucose agar.

It would seem that the glucose is not as efficient as a source of energy as is glycerol. The energy supplied by the latter permits complete utilization of such nutrient substances of the medium as are needed by the organism. With glucose it is otherwise, and the organism is forced to obtain additional energy by breaking down some protein.

The low manometric readings, as seen in table 2, indicated that the respiratory quotient of the organism, when grown on glucose agar, approximated 1, which is the theoretical value for carbohydrates. The analytic values conclusively showed that such was indeed the case. In exper. 1, the corrected real quotient was slightly in excess of 1, whereas the manometric reading was slightly negative (-3 mm.). It is possible that there was an error in the determination of the dissolved CO_2 since the amount found appears to be disproportionately large, as will be seen by examination of table 3.

The average of 3 determinations of the corrected real respiratory quotient was 0.992. This value, it should be pointed out, is higher than that of the apparent quotient because of the large amount of CO_2 taken up by the medium.

In table 6 of Part I will be found a summary of the 3 kinds of respiratory quotients obtained in these experiments with glycerol and glucose agar. It serves to emphasize the extent to which the dissolved CO_2 influences the quotient.

Serum Agar.—Three parallel experiments were made to determine the respiratory quotient of the tubercle bacillus when grown on serum agar. For this purpose, each of 6 sterile, loosely plugged tubes received 5 c.c. of 2% agar. The tubes were then autoclaved at 110 C. for 20 minutes. When cooled to 60 C., each tube received 5 c.c. of rabbit serum which had been kept in the icebox for 4 days. The contents of each tube were at once mixed, and then slanted over night. In the morning, they were inoculated, by means of the spatula, with a culture that was 22 days old. The cotton plug of each was then cut off, flamed and pushed inside the tube. Each jar received 2 of the tubes, and an additional tube containing 5 c.c. of sterile distilled water. To insure the prompt development of full aqueous tension, 5 drops of water were placed on the bottom of each jar. The jars were then sealed in the usual way, attached to manometers, and placed in the hot-room at 37 C. They were equilibrated 10 hours later, and were then allowed to remain undisturbed for 28 days.

The manometers on 2 of the jars (exper. 2 and 3) showed no change in pressure for 14 days. At the end of the 28-day period, they registered only —2 mm. of pressure. The manometer in exper. 1, after a few days, began to indicate a slight negative pressure, which soon became positive, and except for 2 days, remained so till the end. The pressure as observed, from day to day, varied with the barometer, and this indicated that there was a slight leakage into the jar. As a result the analytical values (table 4) for CO_2 and N_2 were low while that for O_2 was high, and hence the respiratory quotients were also somewhat higher than they should have been.

The growth on the serum agar was not nearly as rich as on glycerol agar, or even on glucose agar. Instead of spreading all over the surface, it was confined to isolated masses or colonies which, however, were of good size and typical in appearance. The relative poorness of growth was reflected in the gas changes as found at the close of the experiment.

The results obtained in these experiments are given in table 4. They should be compared, first, with those obtained with glycerol agar, in exper. 3, table 1, which was nearly of the same duration, and likewise had 2 tubes. It will be noted that while the serum agar cultures gave at most 2.5% of CO_2 , those on glycerol agar produced 15%. The total production of CO_2 in the former amounted to only 41 c.c., while with the latter it reached 207 c.c., or fully 5 times as much. The O_2 consumption in the former was 46 c.c. as against 249 c.c. in the latter.

It has been shown that the gas exchange for cultures grown on glucose agar was considerably less than when grown on glycerol agar. The serum agar experiments should be compared with glucose agar

exper. 1, table 2, which also had 2 tubes and was nearly the same in duration. It will be seen that the cultures on glucose agar produced only a little more CO₂ than did those on serum agar, viz., 3.5 as compared with 2.5%. The total yield of CO₂ with the former was 52 c c., while with the latter it was 41 c c. Similarly, the O₂ loss was 50 c c. as against 46 c c.

Attention should be called to table 5 in which a comparison of the gas changes on the 3 mediums will be found. The values given in

TABLE 4
ANALYSES AND QUOTIENTS OF B. TUBERCULOSIS GROWN ON SERUM AGAR

Experiment No.	1	2	3
No. of days.....	28	28	28
No. of tubes.....	2	2	2
Net air volume.....	1843.89	2050.77	1662.98
Barometer.....	742	742	742
Temperature, C.	37.6	37.6	37.6
Corr. obs. man.	-2.0	-2.0
Calc. real man.	-3.7	-5.3	-6.9
Analyses			
CO ₂	1.944	2.125	2.602
O ₂	18.588	18.231	17.562
N ₂	79.468	79.644	79.836
	100.000	100.000	100.000
Corr. analyses*			
CO ₂	1.933	2.108	2.576
O ₂	18.487	18.092	17.389
N ₂	79.04	79.04	79.04
	99.460	99.240	99.005
C c. at 0 C., 760 mm.			
Dissolved CO ₂	6.343	6.755	7.612
Gaseous CO ₂	27.974	33.912	33.607
Total.....	34.317	40.667	41.219
O ₂ loss.....	35.912	46.316	46.741
Quotients	Average		
Apparent resp.	0.785	0.817	0.764
Real resp.	0.743	0.779	0.718
Corr. real resp.	0.904	0.953	0.881
CO ₂	0.217	0.227	0.226

* See footnote to table 1.

tables 1, 2 and 3 have been recalculated to ordinary conditions, namely, 37 C. and 750 mm. pressure per tube of the tubercle bacillus. From the experiments which extended over the same period of time, 26-28 days, it will be seen that serum agar cultures utilized the O₂ in only 135 c c. of air; the glucose agar cultures used up but a trifle more, 149 c c.; while the glycerol agar tubes greatly exceeded these by drawing on more than 800 c c. of air.

These results on respiration serve to emphasize the fact that glycerol agar, as a culture medium, is greatly superior to glucose or serum agar.

When considering the experiments with glucose agar, it was pointed out that the amount of CO_2 dissolved in the medium was considerably in excess of that taken up by the glycerol agar. In other words, while the culture grown on glycerol agar produced practically no alkali, that developed on glucose agar made basic products sufficient to fix a considerable amount of CO_2 . On comparing the quantity of CO_2 dissolved or fixed by the serum agar with that found for glucose agar in exper. 1, table 2, it will be seen that they were about the same, though possibly the serum agar, with less CO_2 output, fixed a relatively larger amount. For purposes of comparison, reference should be made to table 3, in which it will be seen clearly that the amount of CO_2 taken up by 10 c. c. of the serum agar was about one-fifth of the gaseous CO_2 made by one culture; with glucose agar, it was about $1/10$; while with glycerol agar, it was $1/150$ - $1/300$.

It has been shown that the relatively large amount of CO_2 taken up by the medium was an indication of the formation and presence of basic products. The experiments on serum agar cultures developed the further fact that in part at least this alkali was volatile. It will be recalled that in these experiments on the respiratory quotients, each jar received 1 or 2 cultures and also a tube containing 5 or 10 c. c. of distilled water.

The amount of CO_2 taken up by pure distilled water depends on the temperature, the pressure of the overlying atmosphere, and on the partial pressure of the CO_2 present. Since water, at the body temperature takes up out of an atmosphere of pure CO_2 approximately 0.5 volume of the gas, it follows that 10 c. c. of water would take up 5 c. c. of CO_2 . From an air containing 2% of CO_2 , 10 c. c. of water would absorb 0.10 c. c.; from 9% CO_2 air, 0.45 c. c. and for 15% CO_2 air, 0.75 c. c.

On reference to table 3, it will be seen that 10 c. c. of water, in the glycerol agar experiments, actually absorbed somewhat less than the amounts calculated for 9 and 15% CO_2 atmospheres. In those experiments, therefore, the CO_2 was in plain solution. By contrast, it will be noted that in the serum agar experiments the amount of CO_2 taken up by the water was more than 10 times that which should be taken out of an atmosphere containing 2% of CO_2 . It follows that the high CO_2

content of the water in those experiments must be due to the absorption of a volatile alkali, presumably NH_3 .

The effect on the respiratory quotient of the large amount of CO_2 taken up by the medium will be seen in table 4. It is even more marked than with glucose agar (table 2) in which the corrected real respiratory quotient was 0.13, 0.07 and 0.07 higher than the real quotient. With the serum agar medium, the increase was 0.17, 0.14 and 0.16.

The average of the 3 determinations of the corrected real respiratory quotient was 0.904. This was higher than was anticipated for the combustion of serum protein and peptone. There is a possibility of autolytic changes taking place in the serum agar, and if so, these might give rise to some alkali and CO_2 . The proper control to determine the extent of such secondary change, if any, would be to place an uninoculated serum agar tube in the same jar with the inoculated ones.

Plain Agar.—Although the organism could not be grown in air on plain agar, an attempt was made with this medium in the hope of obtaining some evidence of gas exchange. Two tubes, inoculated with the organism, were placed in a jar, which was then evacuated to — 170 mm., after which O_2 was admitted until the pressure reached zero. Analysis showed the presence of 38.77% O_2 and no CO_2 . Another jar, likewise with 2 inoculated tubes, but containing ordinary air, was used as a check.

At the end of 55 days, at 37 C., the growth was extremely slight, if any. There was no difference in the appearance of the tubes in the 2 jars. Analysis of the content in the high O_2 jar showed the presence of 1.64% of CO_2 and 37.35% of O_2 . This slight change in the composition of the initial gas was about what could be expected if uninoculated tubes had been kept in the jar for that length of time. The organism was therefore unable to respire on the plain agar. Failing to utilize the medium as a source of energy, it could not multiply.

It is evident that the organism literally starves in the presence of nutritive substance which it cannot make use of in the absence of a suitable energy supply. Glycerol, and to a less extent, glucose, enables it to assimilate these substances.

OXYGEN REQUIREMENT

The Required Air Volume.—The experiments on the determination of the respiratory quotient reveal the oxygen needs of a good tube culture of the tubercle bacillus. The amount required could not be predicated except in most general terms. It is recognized that the organism is an obligative aerobe, but whether it requires 100 or 200

or 500 c.c. of air was not evident. Indeed, the absence of definite data on this point has not infrequently led to error. There is a general agreement that in the tube culture the tubercle bacillus grows very slowly, requiring 4 to 6 weeks, that the growth may be poor, and that often it fails entirely. As a matter of fact, the growth is quite rapid, very rich and never fails on a good medium when the oxygen requirements, and that of moisture, are satisfied. Under these conditions, a very rich growth can be obtained in less than 14 days (fig. 14).

In the large respiratory chamber, or anaerobe jar, it was possible to determine the amount of oxygen consumed by a good culture during its growth. The fundamental data on this point are given in tables 1 and 2. It seemed desirable, however, to present the results in terms which could be easily understood. With this object in view, the values were recalculated to give the gas exchange per tube of culture at the actual temperature of 37 C., and the average barometric pressure of 750 mm. The volume of air which supplied the amount of O₂ consumed under these conditions was then calculated. The results thus obtained are given in table 5.

TABLE 5
GAS CHANGES PER TUBE OF B. TUBERCULOSIS RECALCULATED TO 37 C. AND 750 MM., MOIST

Agar.....	Glycerol			Glucose			Serum		
Experiment No.	1	2	3	1	2	3	1	2	3
No. of days.....	27	27	26	26	85	85	28	28	28
CO ₂ produced, c c.	145.19	151.73	127.26	32.07	77.23	81.23	21.07	24.96	25.30
O ₂ consumed, c c.	170.86	182.93	153.33	31.25	77.27	85.45	22.05	28.43	28.69
O ₂ consumed represents an air volume of.....	816.34	874.01	732.61	149.31	369.19	408.27	105.35	135.83	137.07

From the formula used when reducing a gas volume to 0 degrees and 760 mm., dry (Part I), the formula for conversion of such values back to any desired temperature and pressure and moist is directly derived. It becomes

$$V = \frac{V_0 (1 + 0.003665 t^{\circ}) 760}{B - T}$$

Substituting, 37 C. for t and 702.92 for $B - T$, where $B = 750$ and $T =$ aqueous tension at 37 C., and solving we obtain the formula

$$V = V_0 \times 1.22782$$

By means of this formula, the values were obtained which are given in table 5.

It will be seen from this table that a single tube culture of the tubercle bacillus, grown on glycerol agar, consumed from 153 to 183 c.c. of oxygen. These amounts of oxygen were obtained from 732 to 874 c.c.

of air. In other words, an actively growing tube culture can consume all of the oxygen out of about $\frac{3}{4}$ of a liter of air. This means that a fair growth may be expected if the oxygen source is restricted to 250 c.c. of air; that a good growth is obtainable with 500 c.c., and that the richest growths call for approximately 1,000 c.c. of air.

In table 5 will be found also the corresponding values for a single tube of the tubercle bacillus grown on glucose agar. It will be seen that the O_2 consumption ranged from 31 to 85 c.c. and that the corresponding air volumes were 149 to 408 c.c. The growth on glucose agar, as pointed out heretofore, was not as rich as that obtained with the glycerol medium. In the same time, 26 days, the organism on glucose agar consumed 31 c.c. of O_2 while on glycerol agar it used up 153 c.c., or 5 times as much.

Tubes Sealed in Flame.—One experiment is given in order to present a clean-cut demonstration of the effect of a limited supply of air in the culture tube. Each of 6 tubes received 10 c.c. of glycerol agar. Two of the tubes were of the usual size (fig. 1 *A*, Part I); 2 were provided with bulbs of medium size and 2 with larger bulbs (fig. 2 *A*, Part I). After inoculation, the tubes were sealed in the blast-lamp. The volumes of air in the 3 sets of tubes approximated 40, 90 and 140 c.c. The tubes were then photographed and placed at 37 C. for 27 days, when they were again photographed. The tubes were returned to the hot-room, where they remained for 5 months without showing any further increase in growth.

Fig. 1 shows the tubes at the time of inoculation. It serves as a control for fig. 2, which, as stated, was taken 27 days later. It will be seen at a glance that the straight tubes, nos. 1 and 2, with about 40 c.c. of air, gave no evidence of growth. The tubes with medium bulbs, nos. 3 and 4, and an air capacity of about 90 c.c., showed a slight but distinct growth, while the tubes, with large bulbs, nos. 5 and 6, with about 140 c.c. of air, exhibited a fair growth. The 3 pairs of tubes were rated as —, 1 + and 3 +, whereas control tubes, with "holed" sealing-wax were very rich and scored 10 +. The growth was therefore proportional to the air supply. The tubes with 140 c.c. of air gave but a fair growth, thus demonstrating that 3 to 4 times that volume of air was necessary to obtain a really rich culture.

Tubes Closed with Sealing-Wax.—It is necessary, in order to prevent desiccation of the medium, to close in some way the end of the culture tube. Sealing-wax has been used for this purpose with variable results. In our work, frequently, good cultures were obtained under a cap of sealing-wax, and then, again, for apparently no reason, growth was poor or failed entirely. It was essential to know the cause of this peculiar behavior, and much effort was expended in that direction. The assumption that sealing-wax allowed sufficient diffusion of gases to permit growth was found to be erroneous. The cap of sealing-wax when properly applied is air-tight.

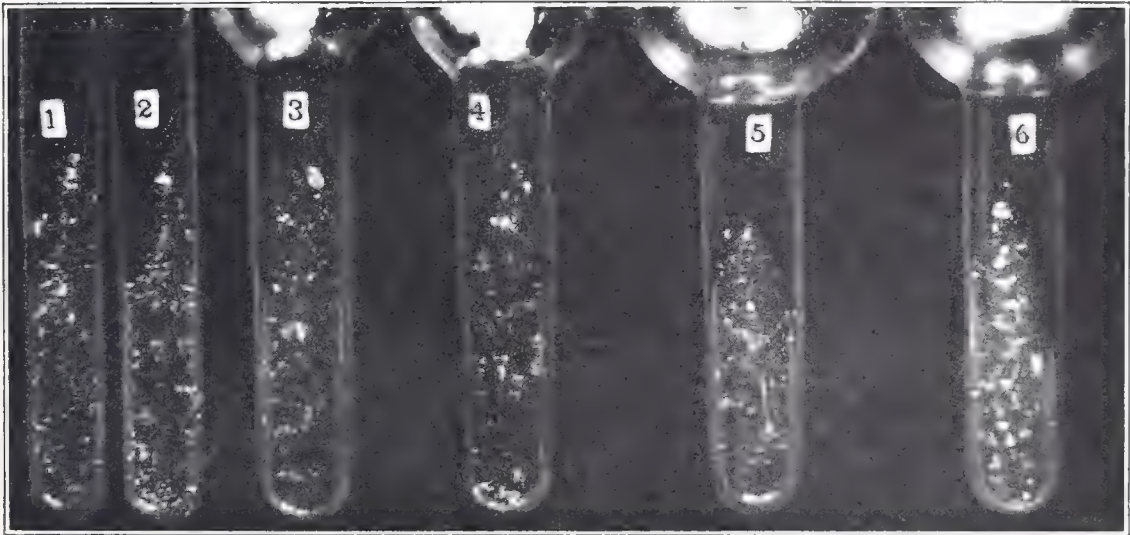


Fig. 1.—Growth of the tubercle bacillus in tubes which were sealed in the flame. Appearance of the tubes immediately after inoculation. Air capacity was 40, 90 and 140 c.c.



Fig. 2.—Appearance of the tubes shown in fig. 1 after 27 days at 37 C. The growth was proportional to the air volume, and ceased when the oxygen was exhausted. No further increase occurred, although the tubes were kept for 60 days longer at 37 C.

It can easily be determined whether or not the sealing-wax is air tight. For this purpose the tube is cut in two, a short distance above the agar slant. The end piece is attached to the rubber stopper, to which glycerol has been applied, on the end of a manometer. The latter is connected with a suction pump by means of cock 3. The tube is then evacuated to -200 mm. or more, and the pump is disconnected. When a tube which shows no growth is tested in this manner, it will be found to hold this pressure indefinitely. On the other hand, with a tube in which there is a good growth, scarcely any negative rise can be obtained, or if there is one, it drops the moment the action of

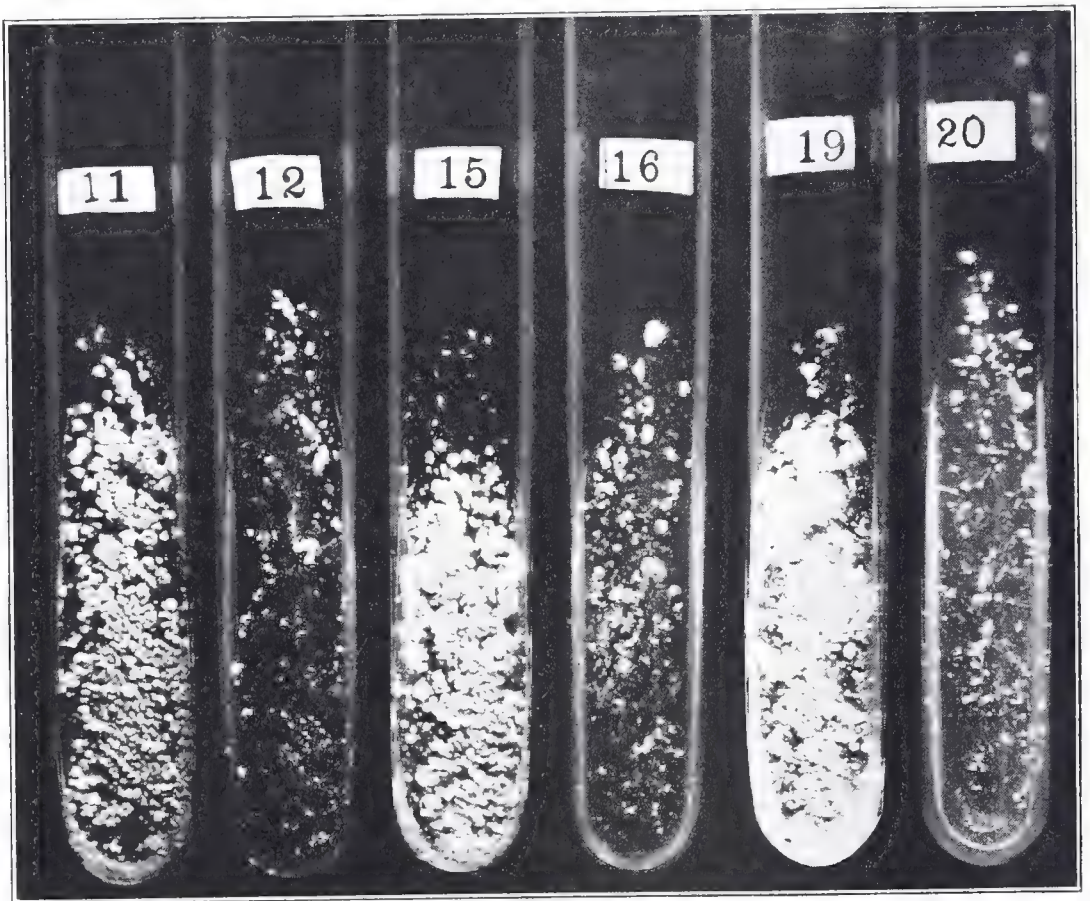


Fig. 3.—Growth of the tubercle bacillus under sealing-wax, 35 days at 37°C . On the 21st day, when no growth was in evidence, a hole was made with a hot platinum wire in the sealing-wax of nos. 11, 15 and 19. Result, a rich growth in the next 14 days. Nos. 12, 16 and 20 were not holed, and gave practically no growth.

the pump is stopped. Growth is, therefore, the result, not of diffusion, but of leakage through minute openings along the lip of the culture tube.

In one of the early experiments which concerned the question under consideration, 20 tubes were inoculated and closed with sealing-wax. At the end of 21 days, no growth was discernible in any of the tubes. On that day, a small hole was made through the sealing-wax on the odd numbered tubes by means of a hot platinum wire. Within a week the "holed" tubes showed

good growth, whereas those that were not perforated remained unchanged. In 2 weeks, there was a uniform and striking contrast in the two sets of tubes. Fig. 3 is a photographic reproduction of 3 pairs of these tubes. It shows in a striking manner the importance of providing an adequate air supply.

It may be added here that these experiments with sealing-wax were made before the full oxygen needs of the organism had been established by means of the anaerobe or respiratory jar. They showed that the successful culture under sealing-wax had been the result of an accident, the presence of one or more small openings through which air freely entered the tube. It remained therefore to apply this fact in the routine of cultivation.

The following procedure can therefore be recommended as giving the best possible cultures of the tubercle bacillus. The medium, which contains 1% agar and 5% glycerol, is inoculated after slanting. The cotton plug is then cut off, flamed, and the tube is closed with a cap of sealing-wax. By means of a white-hot platinum wire, a hole less than 1 mm. in diameter is made through the sealing-wax. At 37 C. the growth is visible on the 3rd or 4th day; it is good on the 7th day, and very rich on the 14th day. There is no failure with a viable culture. Little desiccation is in evidence before the 3rd or 4th week. Moisture, however, is another important factor which will be considered later.

It was of some interest to know how much CO₂ was present in an actively growing "holed" culture. The medium in 3 tubes, with side-arms (fig. 2 C, Part I) was inoculated, after which the tubes were closed with sealing-wax. A fairly large hole was made through the wax by means of a hot wire of No. 17 gage. The tubes were attached, by means of the side-arm, to manometers and placed at 37 C. When it was desired to examine the gas content, the hole in one of the tubes was closed with sealing-wax, after which the sample of gas was drawn in the usual way and analyzed. A very good growth was present on the 7th day and rapidly increased in volume. One tube was examined on the 7th day, another on the 14th and the third on the 21st day. It will be seen from the following that the CO₂ content increased with the mass of the culture, the gas escaping less rapidly than it was being formed.

	7th Day	14th Day	21st Day
CO ₂	1.33	3.46	9.44
O ₂	19.56	17.55

In a tube which is not sealed in some way, no matter how solid the cotton plug may be, desiccation takes place, and hence only a slight growth is obtained. The CO₂ which is formed in such a tube rapidly diffuses outward. Thus, Moore and Williams¹ found no CO₂ when they examined, at the end of 14 days, a tube which was inoculated with the avian tubercle bacillus and left unsealed. A similar experiment in our hands, on the 15th day, gave only 0.22% of CO₂. The tube in this case was 35 x 190 mm. and contained 30 c.c. of glycerol agar. It was closed with as solid a cotton plug as it was possible to make. A tail-cock with an arm bent at right angles was fused to the side of the tube. Before drawing the sample for analysis, a rubber stopper to which glycerol had been applied was pushed into the mouth of the tube.

Tubes Closed with Paraffin.—It is a common practice to seal cultures of the tubercle bacillus either by dipping the cotton plug into melted paraffin,

¹ Biochem. Jour., 1909, 4, pp. 177-190; 1911, 5, pp. 181-187.

or by pouring the latter on top of the plug. The result is a seal which not only prevents desiccation but also retards and may even prevent growth. The condition parallels exactly that which has been shown for sealing-wax. Paraffin can make an effective air-tight seal. Thus, if paraffin is poured on top of a firm cotton plug so as to form a layer, about 15 mm. deep, and the tube is then connected with a manometer and evacuated to -700 mm., this vacuum will be held perfectly at room temperature. At 37°C ., however, the paraffin softens somewhat so that, after some time, the plug is forced into the tube and some leakage develops.

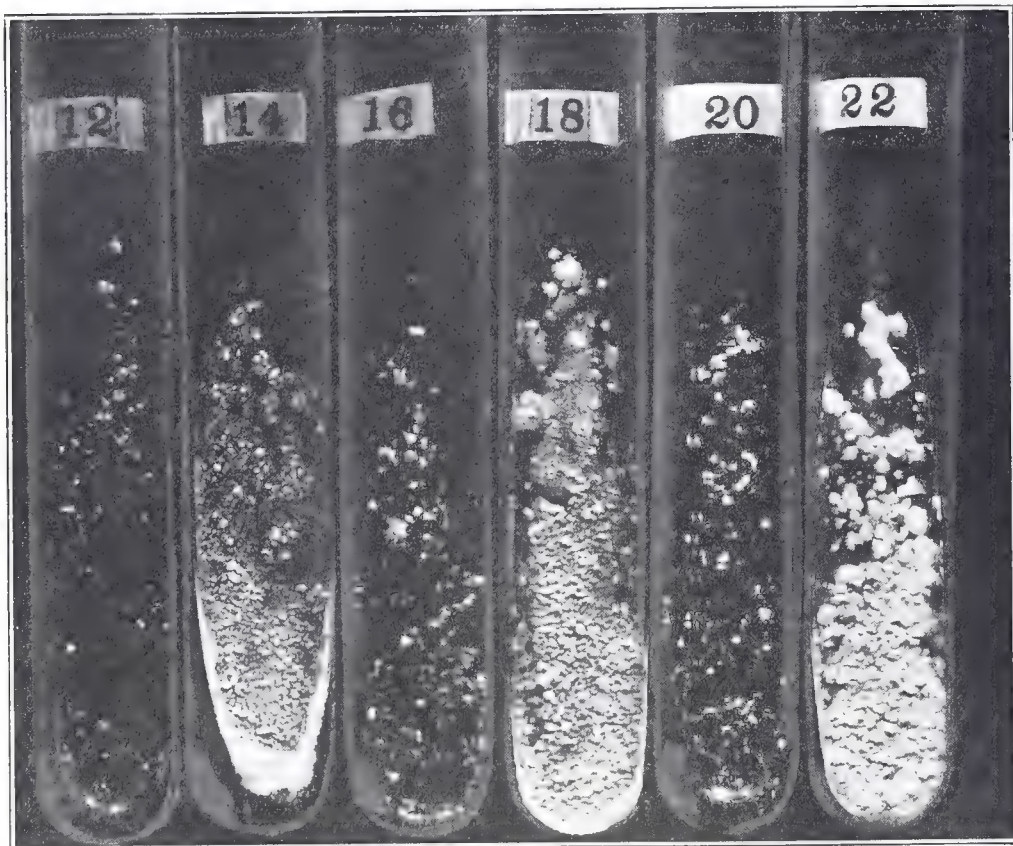


Fig. 4.—Growth of the tubercle bacillus under different seals, 21 days at 37°C . Nos. 12 and 14 were sealed with paraffin, but in no. 14 a finely drawn-out capillary was inserted between the paraffined plug and the wall of the tube. Nos. 16 and 18 were closed with rubber stoppers, but no. 18 had a drawn-out capillary inserted into the rubber stopper. Nos. 20 and 22 were closed with sealing-wax, but no. 22 was holed by means of a hot platinum wire.

The effect of the paraffin seal on the growth of the tubercle bacillus can best be shown by reference to fig. 4. Tube 12 was sealed by dipping the cotton plug into paraffin. The plug of tube 14 was treated in like manner, but a fine drawn-out capillary was placed between the wall of the tube and the paraffined plug. Fig. 4 shows the appearance of the tubes at the end of 21 days. Tube 12 with the solid paraffin plug gave no growth, whereas No. 14, with the fine capillary, developed a good culture. The capillary,

like the hole in the sealing-wax in tube 22, admits the necessary supply of air and thus favors growth. Tube 12 may likewise be compared with tube 20, which was closed by sealing-wax.

Tubes Closed with Rubber Stoppers.—A dry rubber stopper when inserted into a tube, as a rule, will not hold a vacuum. If, however, glycerol is applied, it will be found to be air tight. In the experiment shown in fig. 4, tube 16 was provided with a solid stopper which was treated in the usual way with glycerol. A similarly treated, perforated stopper, provided with a drawn-out capillary, was used for tube 18. Tube 16, like tubes 12 and 20, showed no growth in 21 days, whereas its companion with the capillary tube 18 gave an abundant culture.

As in the case of the "holed" sealing-wax culture, it was desirable to have some definite information as to the amount of CO_2 which could be expected in a tube closed with a perforated rubber stopper. An *h*-tube, with a freshly inoculated culture, was therefore provided with a rubber stopper into which was inserted the arm of a tail-cock (fig. 13, Part I). The cock was left open, thus providing an opportunity for the escape of CO_2 . On the 21st day, the cock was closed and a sample of the air in the tube was drawn and analyzed. It was found to contain 5.93% of CO_2 and 15.49% of O_2 . This result, therefore, compares very well with that obtained with the "holed" sealing-wax cultures.

Rubber caps have been extensively used for closing tubes in order to prevent evaporation. When made of thin rubber, there is every reason to believe that some gas diffusion will take place. The rate of such diffusion by different gases has been pointed out in Part I. However, no actual tests have been made, as with other seals, of the extent to which inhibition of growth takes place. There is every reason to believe that a pin-hole in the rubber cap will be found to be advantageous.

It is evident from what has been said that if a culture tube is actually sealed, whether in the blast-lamp, or with sealing-wax, paraffin or rubber the result is the same—a failure to produce growth. The limited oxygen supply does not suffice to permit a visible development. When, however, accidentally an imperfect seal is made, growth results. Consequently, the method given above, of intentionally providing an air supply by means of a minute hole in the sealing wax, is the one to be preferred for the cultivation of the tubercle bacillus.

The idea of providing a plentiful and certain supply of air is not a new one. As long ago as 1898, Smith² made use of a test-tube with a ground glass cap which was provided with a narrow tube similar to fig. 2*B*, Part I. About the same time, Novy³ employed a cork stopper into which was inserted a fine capillary tube, and he was thus able to obtain unusually fine growths.

² Tr. Am. Physicians, 1898, 13, p. 417.

³ Laboratory Work in Bacteriology, Ann Arbor, Wahr, 1899, p. 315, fig. 54.

MANOMETRIC CHANGES

The study of the gas changes within culture tubes which were attached to manometers in the manner heretofore given was an essential preliminary to all of the work on the respiration of the tubercle bacillus. When the main facts were once established it was possible to attack successfully the various phases of the problem as they arose. It is therefore of interest to consider the results obtained by this method of investigation.

Table 6 gives the manometric results in the sixth experiment of that kind. It would be of no particular value to reproduce the records of the other experiments unless it were to show the difficulties which had to be overcome. First of all, the table is intended to show the effect of different seals on the pressure changes. The effect on the composition of the contained gas will be developed in table 8.

In all experiments of this kind, it is desirable to know the composition of the air in the tubes at the beginning of the experiment. Hence in this case, a tube (no. 16) was inoculated and attached as were tubes 1, 2 and 3. Immediately after the equilibration of all of the manometers in this experiment, this control tube was analyzed and was found to contain 0.12% of CO_2 , and 20.89% of O_2 .

Tubes 3, 6, 9, 12 and 15 were uninoculated controls. They remained sterile throughout the experiment. It is worthy of note that these tubes showed a slowly progressive rise in negative pressure. The change was least marked in no. 12, which was an all glass seal. Hence it follows that some of the negative pressure observed in these controls is due to oxidative changes in the agar medium. The rubber stopper and the sealing-wax, as well as the cotton plug and glycerol, must be considered as capable of undergoing some oxidation. We have here a simple demonstration of a well-known fact, one to which Pasteur called attention 60 years ago, that organic substances in the presence of oxygen and moisture undergo slow oxidation. Definite proof of this statement will be found in the results of the analyses given in table 8.

In discussing the subject of aqueous tension in Part I, it was pointed out that agar was hydrophilic and as such tended to lower the water tension within a tube. The result of this would be a negative pressure. The cotton and the glycerol can conceivably act in the same way. Hence, a part of the observed negative pressure in these control tubes may be due to an effect of this kind. The major part of the negative pressure is clearly due to the oxidative changes within the sterile tubes.

Attention may next be called to the inoculated tubes—1, 2, 4, 5, 7, 8, 10, 11, 13 and 14. It will be seen that the negative pressure rose in all, but it did so more rapidly in some than in others. This inequality was frequently observed and the obvious explanation was in the unequal number of organisms in the original inoculation. Although care was taken to transfer approximately equal amounts of culture to each tube, differences in the amount of the inoculum were unavoidable.

It will be noted further that the negative pressure reached a maximum on about the 4th to the 6th or 7th day, with the exception of no. 11, in which the change was the slowest of all. In the case of tubes 2, 5, 8, 11 and 14, which were not analyzed until the 28th day, this maximum, once reached, persisted until the end. The analytical results showed that the oxygen had practically disappeared when the maximal negative pressure was reached (table 8); in other words, when the oxygen was gone, respiration ceased. The fact that the maximal negative pressure, once reached, persisted during the following 3 weeks of the experiment was a good indication of the absence of leakage.

This experiment, it may be stated here, was planned with the object of ascertaining the efficiency of the different seals and connections employed. The all-glass seals, as used for tubes 10, 11 and 12, showed no particular advantage over the simple closure with sealing-wax and the use of the no. 25 rubber stopper connector (tubes 1, 2 and 3). It will be seen that the negative pressure in tubes 2, 5, 8 and 11, which were unopened for 28 days, was essentially constant and practically the same in all of the 4 manometers. The fall of about 7 mm. in the pressure of tube 14 was almost too gradual to be due to leakage. When leakage does occur, the Hg column promptly falls to within a few mm. of zero.

A noteworthy fact observed in connection with these unaerated tubes was the absence of any visible growth. Whatever multiplication took place did so before the negative pressure had reached its maximum, i. e., before the O_2 was consumed. There being no further supply of O_2 , growth necessarily ceased. The adverse condition, due to the absence of O_2 and the presence of CO_2 , often caused the death of the organism by the end of the 4th week.

Aerated Series.—Tubes 1, 4, 7, 10 and 13 were analyzed on the 6th or 7th day, as soon as it seemed certain that the maximal negative pressure had been reached (tables 6 and 8). The 5 manometers were then attached, by means of cocks no. 3, to a glass rake, or multiple T (fig. 7, Part I), which was connected with the water pump in the manner described in Part I. The tubes were evacuated to 650 mm., and air was admitted through a tube filled

TABLE 6
MANOMETRIC READINGS OF CULTURES OF TUBERCLE BACILLUS WITH VARYING SEALS AND CONNECTIONS

Tube.....	Sealing Wax R. S. Connect.				Glass Seal R. S. Connect.				S. W. + Hg Seal R. S. Connect.				Glass Seal G. S. Connect.				Sealing Wax G. S. Connect.				Temp. C.
	1	2	3C	4	5	6C	7	8	9C	10	11	12C	13	14	15C						
Days Hrs.																					
0-1*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	37.6		
9	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	37.2		
1	8	8	7	12	8	2	8	6	6	7	9	2	1	3	0	0	0	0	37.4		
2	33	13	7	18	10	2	9	7	7	12	3	3	5	11	0	0	0	0	37.2		
2	45	57	7	21	17	2	18	7	7	20	3	3	8	14	0	0	0	0	37.4		
3	39	30	10	29	23	4	23	8	8	25	3	3	11	17	0	0	0	0	37.9		
3	69	39	10	33	26	4	36	9	9	31	4	4	16	21	0	0	0	0	37.3		
4	81	44	10	41	31	4	43	9	9	40	4	4	20	24	0	0	0	0	37.3		
4	95	45	10	45	34	5	48	9	9	49	4	4	22	29	0	0	0	0	37.8		
4	105	49	10	51	40	5	52	9	9	50	5	5	32	31	0	0	0	0	37.5		
5	117	49	11	55	45	6	55	11	11	54	6	6	37	37	0	0	0	0	38.0		
5	129	53	11	58	48	6	55	11	11	54	6	6	41	40	0	0	0	0	37.6		
6	141	49	14	61	50	6	58	11	11	58	6	6	44	43	0	0	0	0	37.8		
6	153	55	13	57	54	6	55	11	11	60	6	6	48	46	0	0	0	0	37.7		
7	160	55	13	60	54	6	56	11	11	60	6	6	48	46	0	0	0	0	37.7		
0-2																			37.3		
8	3	55	16	4	54	8	9	13	13	7	6	6	16	50	2	2	2	2	37.9		
8	21	55	16	19	54	8	60	14	14	18	6	6	16	52	2	2	2	2	37.5		
9	33	53	17	33	54	8	60	14	14	32	6	6	28	54	2	2	2	2	37.6		
9	45	54	17	44	54	8	58	15	15	45	6	6	38	54	4	4	4	4	37.3		
9	57	53	17	48	54	8	58	15	15	50	6	6	39	55	4	4	4	4	37.9		
9	69	56	17	47	54	9	58	16	16	48	6	6	40	53	4	4	4	4	37.9		
10	81	54	18	46	52	9	57	17	17	48	6	6	40	54	4	4	4	4	37.9		
0-3																			37.5		
11	11	54	18	19	53	10	17	17	17	23	7	7	15	54	4	4	4	4	37.8		
12	29	54	19	37	54	10	34	17	17	39	7	7	30	54	4	4	4	4	38.4		
12 1/2	41	53	18	41	53	10	38	18	18	39	7	7	31	53	4	4	4	4	38.4		
13	53	53	20	41	54	10	40	19	19	40	8	8	33	53	5	5	5	5	37.3		
13	65	54	20	40	54	11	38	19	19	39	8	8	32	53	5	5	5	5	37.3		
0-4																			37.6		
14	3	54	20	8	54	11	2	19	19	5	8	8	3	53	5	5	5	5	37.8		
14	14	54	21	18	54	10	18	20	20	22	8	8	17	53	6	6	6	6	37.8		
15	27	56	24	37	54	13	35	23	23	36	7	7	32	53	7	7	7	7	37.6		
15	38	56	23	39	54	11	36	21	21	36	7	7	32	53	7	7	7	7	37.6		
16	47	56	23	42	54	11	38	21	21	36	7	7	32	53	7	7	7	7	37.6		

17	0-5	0	55	24	-2	54	13	0	60	20	-4	58	7	-3	51	7	7	38.2
	12	-15	54	24	21	56	13	-18	58	22	24	57	8	18	50	9	9	37.3
	24	30	55	24	35	55	14	32	57	22	35	57	8	32	50	9	9	37.8
	36	34	55	25	36	54	13	35	58	22	36	57	8	31	50	9	9	37.6
18	0-6																	
	11	-11	55	25	-17	54	13	-14	57	22	-22	57	8	-15	50	9	9	37.6
	19	-27	56	27	31	56	12	23	60	23	34	57	8	27	50	9	9	37.8
	35	25	53	25	30	54	14	19	57	22	33	54	6	26	48	9	9	38.8
20	47	25	53	26	31	54	13	29	58	23	34	55	8	27	50	11	11	37.7
	58	27	54	27	32	55	13	32	58	23	33	55	8	26	50	12	12	37.3
	61	26	54	27	35	55	13	35	58	23	34	55	8	26	50	12	12	37.3
																		...
21	0-7																	0
	6	-4	55	29	-9	57	17	-8	60	24	-11	56	9	-9	50	14	14	36.8
	18	24	55	29	29	56	13	27	58	25	31	56	8	27	50	14	14	37.4
	42	26	55	30	34	57	15	31	60	25	31	56	6	27	48	15	15	37.2
23	0-8																	3
	6	-6	55	30	-9	57	15	-9	60	25	-10	56	7	-10	49	15	15	37.3
	18	22	55	30	25	57	14	25	60	25	18	56	7	27	49	15	15	38.0
	31	24	56	31	28	54	14	28	58	25	18	55	7	27	49	17	17	37.3
25	54	24	55	31	25	54	14	26	58	25	18	55	8	26	49	17	17	37.5
	54	24	56	33	27	55	14	26	57	25	18	55	8	25	48	18	18	38.0
	66	24	56	33	24	54	14	24	56	25	15	55	8	23	48	18	18	38.0
	91	21	56	33	24	54	14	23	57	25	13	55	8	21	48	19	19	38.0
27	0-9																	...
	6	-6	55	30	-9	57	15	-9	60	25	-10	56	7	-10	49	15	15	37.3
	18	22	55	30	25	57	14	25	60	25	18	56	7	27	49	15	15	38.0
	22	27	56	33	32	55	14	30	59	25	30	55	8	28	48	19	19	37.3
Growth	4 days	?	?	0	?	?	0	?	?	0	?	?	0	?	?	0	0	17+
	10 days	2+	+	0	2+	+	0	2+	+	0	2+	+	0	2+	+	0	0	18+
	14 days	4+	+	0	4+	+	0	4+	+	0	4+	+	0	4+	+	0	0	19+
	21 days	7+	+	0	7+	+	0	7+	+	0	7+	+	0	7+	+	0	0	20+
28	28 days	10+	1+	0	10+	1+	0	10+	1+	0	10+	1+	0	10+	1+	0	0	21+
																		22+
																		23+
																		24+

* The numeral after the zero hour, in this column, indicates the number of the air refill, at that point, for Tubes 1, 4, 7, 10 and 13.
+ Tubes Nos. 17 and 18 were unattached controls closed with sealing-wax.

with sterile cotton. This operation was repeated 5 times, so as to replace all of the gas in the culture tubes with normal air. The manometers, with the attached tubes, were then replaced at 37 C., and after an interval of 3 hours, they were equilibrated and the cocks closed. The air in one of the tubes, No. 4, was then analyzed as a control. It may be added that this aeration of the culture tubes was effected without the slightest evidence of contamination.

On reference to table 6, it will be seen that the negative pressure now appeared earlier than at the start of the experiment. Likewise, the maximum was reached in a shorter time. This fact is explained by the increased number of active tubercle bacilli: that is to say, some multiplication had already taken place although visible evidence of growth was either absent or uncertain. Equally interesting was the fact that the maximal negative pressure now reached was lower by about 10 mm. than it was before. The analyses furnish the explanation for this apparent inconsistency (table 8). They show a higher percentage of CO₂ than did the previous analyses, while the oxygen, as before, is practically gone. The lower values for CO₂ (11-13%), in the first period, were due to absorption of this gas by the medium, and this necessarily caused a corresponding negative pressure. In the second period, the medium was nearly saturated, and hence there was little or no loss due to solution of CO₂ in the medium. The CO₂ content was at 15-16%. During the subsequent aeration periods it rose to 17%.

As soon as the negative pressure in this second period reached its maximum, the tubes were analyzed; then, as before, they were evacuated and refilled with filtered air and a control analysis made. This operation of frequent analysis and refilling of these 5 tubes was repeated so that all told 9 aerations took place in 28 days, the duration of the experiment. This number of refills could have been easily doubled, because after the second period the growth became visible, and from then on it rapidly increased in mass. This growing, respiring mass was soon able to bring about a maximal negative pressure in 24 hours or less; in other words, a good growth once established consumed in less than a day practically all of the oxygen contained in the tube. Comparing this with the rate at the start, it will be seen that the speed of oxygen utilization has been increased at least six-fold.

The speed at which the negative pressure rises in the later aerations can best be seen by making hourly examinations. As far as was possible, this was done with the 9th aeration set; the results are given in table 7. Apparently, there was a latent period of 2 hours, after which the rate increased to about 1.5 mm. per hour. The maximum of about -30 mm. was reached at about the 20th hour.

The analyses showed that the oxygen was then reduced, on an average, to 0.5 %, while the CO₂ production was close to 17%. The slightly lower values obtained in tubes 1, 4 and 7 for CO₂ might be ascribed to the absorption of some of this gas by the rubber stopper connectors, but if so, the manometers should indicate a higher negative pressure than that noted in nos. 10 and 13. Slight irregularities of this kind are unavoidable, since in the process of evacuation there may be an unequal loss of CO₂ by the medium in the different tubes.

Growth.—The record of the growths as observed at different intervals is given at the bottom of table 6. Fairly definite indications of beginning multiplication was often noted as early as the 4th day, when numerous pin points or colonies with delicate thin halos could be seen in some of the tubes. In the unaerated cultures, some of these points increased to the size of pin heads, 1 to 2 mm. in diameter, but otherwise the growth was inappreciable even at the end of 28 days. At the end of that time, the growth mass was hardly one-tenth or even one-twentieth of that in the aerated cultures.

TABLE 7

DETAILS OF THE NINTH AERATION TEST, IN TABLE 6, SHOWING THE RAPIDITY IN DEVELOPMENT OF NEGATIVE PRESSURE AND THE FINAL RESULTS OF ANALYSIS

No. of tube.....	1	4	7	10	13
Pressure at: Hrs.					
0.....	0	0	0	0	0
1.....	0	0	0	0	0
2.....	0	0	0	0	0
3.....	-2	-2	-2	-2	-2
4.....	5	5	5	6	4
5.....	6	6	6	7	5
6.....	7	7	7	8	6
16.....	23	27	24	26	25
18.....	26	29	28	27	26
19.....	26	32	28	29	26
20.....	-27	-32	-30	-30	-28
Tubes then analyzed gave					
CO ₂	16.87	16.68	16.54	17.35	17.09
O ₂	0.68	0.41	0.72	0.36	0.36
In the first test these tubes gave a maximal pressure of.....	-49	-58	-55	-58	-48
in hours.....	95	141	129	153	165
and analysis gave					
CO ₂	13.19	12.18	12.75	11.40	12.67
O ₂	0.31	0.05	0.20	1.31	0.96

The aerated cultures, on the other hand, responded to the air treatment by a prompt and continued increase in the mass. The richness of this growth, compared with that in the unaerated tests, can best be seen in the illustration of 5 pairs of these tubes (fig. 5).

At the conclusion of this experiment, aerated tubes 1, 4, 7, 13 and 10 were evacuated and filled with 20, 30, 40, 50 and 95% of O₂, respectively. These well developed, aerated cultures rapidly disposed of the increased amounts of O₂ thus supplied, a fact which could be inferred from the rapid and high manometric response which in tube 10 reached -151 mm. Confirmation of this was given by the analyses which showed high yields of CO₂ (up to 80%) corresponding reasonably to

the amount of O_2 consumed (tests 10 and 11, table 8). These tests proved that the tubercle bacillus could utilize O_2 , even in 100% concentration.

Unaerated tubes 2, 5, 8, 11 and 14 were treated at the same time and with similar concentrations of O_2 as those just mentioned. The results were equally interesting but totally different. They confirmed the fact, repeatedly observed in previous experiments, that the tubes which were unaerated for a period of 28 days, when subsequently refilled with air or oxygen gave little or no manometric response. This fact demonstrated

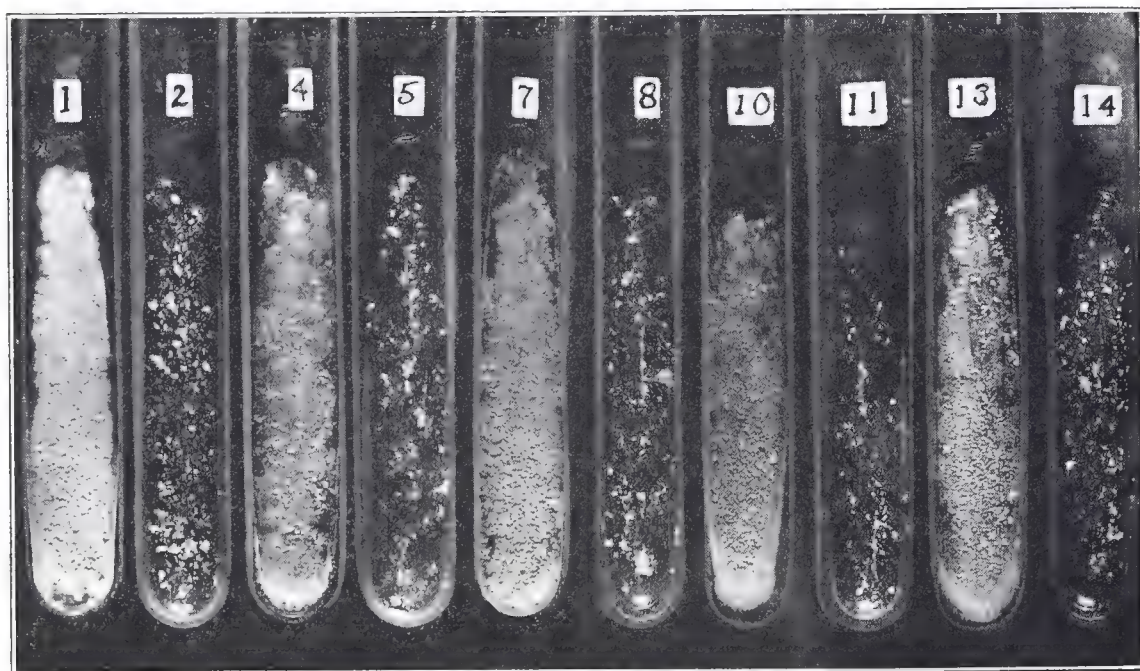


Fig. 5.—Effect of aeration on the growth of the tubercle bacillus, 28 days at 37 C. These tubes were attached to manometers by means of the side-arms. Nos. 1, 4, 7, 10 and 13 were analyzed and aerated 9 times. Result, rich growth. Nos. 2, 5, 8, 11 and 14 were not aerated and hence gave practically no growth. For method of closure and attachment, see table 6.

that the inoculum was dead. In other words, deficient aeration, which means absence of O_2 , brings about a cessation of growth. This condition, together with the presence of CO_2 , results in a slow but sure destruction of the tubercle bacillus.

In table 8 are given the results of the analyses of the 15 tubes, the manometric behaviors of which have been presented in table 6. The aeration series demonstrates the rapidity and completeness of the removal of oxygen in these tests. Equally striking is the regular and uniform

TABLE 8
RESULTS OF ANALYSES OF THE AERATED AND UNAERATED CULTURES OF B. TUBERCULOSIS AND OF UNINOCULATED CONTROLS LISTED IN TABLE 6

Aerated Series			Tube 1			Tube 4			Tube 7			Tube 10			Tube 13		
Closed with.....			S. W. + R. S. C.*			G. S. + R. S. C.			S. W. HgS + R. S. C.			G. S. + G. C.			S. W. + G. C.		
Test Per.	Dur. Hrs.	Bar. Init.	Corr. Man.	CO ₂	O ₂	Corr. Man.	CO ₂	O ₂	Corr. Man.	CO ₂	O ₂	Corr. Man.	CO ₂	O ₂	Corr. Man.	CO ₂	O ₂
1	160	748	-50.3	13.19	0.31	-60.9	12.18	0.65	-56.7	12.75	0.20	-60.6	11.40	1.31	-49.3	12.67	0.96
2	84	746	41.1	16.07	0.23	45.6	15.49	0.12	46.6	15.93	0.03	48.5	15.63	0.04	42.1	16.04	0.19
3	70	749	38.0	16.05	0.30	41.6	15.22	0.38	38.5	16.70	0.04	38.4	16.55	0.16	32.9	16.64	0.25
4	48	755	38.0	16.48	0.0	42.6	16.12	0.10	38.5	16.28	0.02	36.4	16.13	0.16	32.9	16.78	0.14
5	48	749	34.9	16.51	0.40	36.5	16.41	0.27	35.4	16.69	0.14	36.4	16.51	0.31	31.8	16.76	0.28
6	60	741	26.7	17.17	0.18	35.5	16.97	0.17	35.4	16.27	0.15	34.4	17.30	0.13	26.7	17.31	0.20
7	45	746	26.7	17.20	0.13	32.4	16.93	0.12	31.4	17.01	0.18	31.3	17.13	0.08	25.7	17.27	0.11
8	96	742	22.6	17.06	0.68	24.3	17.57	0.0	24.3	17.56	0.28	12.1	18.19	0.12	20.5	17.86	0.54
9	20	740	27.7	16.87	0.68	32.4	16.68	0.41	30.4	16.54	0.72	30.3	17.35	0.36	28.8	17.09	0.36
10	90	735	22.6	18.57	0.31	63.9	23.19	0.21	64.8	37.36	0.14	151.6	80.81	1.25	76.1	44.61	1.16
11	72	751	28.8	17.00	0.15	60.9	25.35	0.19	73.9	37.28	1.39	131.4	81.26	1.09	73.0	46.01	0.51
Unaerated Series			Tube 2			Tube 5			Tube 8			Tube 11			Tube 14		
1	28 d	748	56.7	12.47	0.40	57.4	12.78	0.04	59.9	12.28	0.07	52.9	13.52	0.04	48.6	13.93	0.18
Uninoculated Controls			Tube 3			Tube 6			Tube 9			Tube 12			Tube 15		
1	28 d	748	33.4	1.46	15.48	14.3	0.55	18.07	25.3	0.83	15.57	8.1	1.06	18.82	19.2	2.54	15.40

* The abbreviation refers to "sealing-wax and rubber stopper connection" as written out in full in table 6, where will be found the key to the others.

production of about 17% of CO₂. No attempt was made in these tests to determine the amount of CO₂ taken up by the medium. A good indication, however, of the extent to which CO₂ was taken up, can be obtained by comparing the percentages of CO₂ found in the tubes at the end of the first period, whether it was 6 or 28 days, with those found in the aerated series. The difference of about 4 to 5% in these tests was chiefly due to the solution of CO₂ in the medium.

TABLE 9
MANOMETRIC READINGS OF CULTURES OF TUBERCLE BACILLUS WITH VARYING SEALS, AT 37 C.*

Closed with.....		Paraffin				Rubber Stopper			Glass Seal		
Tube.....		1	2	10	3	4	5	6	7	8	9
Days	Hrs.										
	0	0	0	0	0	0	0	0	0	0	0
	11	0	0	0	0	0	0	0	-3	-2	0
1	35	-13	-4	-10	-6	-9	-6	-6	12	13	-11
2	59	25	8	18	13	20	17	15	20	16	18
3	83	34	15	25	21	28	25	22	26	23	26
4	107	45	22	30	29	41	34	33	32	29	35
5	131	52	27	32	36	51	40	40	36	33	41
6	156	58	37	36	47	65	50	50	43	38	46
7	180	60	42	38	49	73	56	56	45	39	48
8	204	62	46	38	50	81	58	63	51	42	48
	0										
9	16	-3	50	40	53	-5	60	63	-2	45	49
10	40	20	50	31	54	37	61	63	8	47	50
11	64	8	38	29	54	56	62	66	12	48	50
	0										
12	16	-3	-2	+5	54	63	63	66	22	51	50
13	42	12	5	-1	54	66	63	67	29	54	52
14	64	37	23	5	53	67	66	65	34	54	50
15	88	42	42	15	55	64	67	66	36	54	50
16	112	41	45	20	55	66	67	67	40	54	50
17	144	43	48	30	55	69	68	68	43	54	50
	0										
18	16	..	-8	35	55	-5	68	68	+5	55	52
19	40	..	36	38	54	24	71	71	-1	55	52
20	64	..	38	50	54	45	73	77	16	57	54
21	88	..	36	47	54	57	74	77	19	55	52
22	112	..	37	54	54	61	75	77	32	57	52
	0										
23	16	..	-12	0	55	-14	74	70	+3	55	51
24	40	..	38	-15	56	46	74	72	-9	57	53
25	64	..	38	28	56	54	76	73	18	58	55
26	88	..	36	37	56	54	75	72	24	56	53
27	113	..	37	46	56	57	76	74	36	58	57
28	144	..	41	47	58	64	76	77	44	58	57
Growths.....		2+	5+	2+	?	5+	?	?	2+	?	?

* These tubes were joined to the manometers with No. 25 rubber stoppers. They were sealed as indicated above.

Successive aerations of tubes, as in this experiment, gives some information as to the total gas exchange which must take place in order to obtain a good culture of the tubercle bacillus. The average capacity of the empty culture tube was 56 c.c. With 10 c.c. of the medium this

was reduced to 46 c.c., but since the right arm of the manometer had a free space of about 3 c.c., it follows that the total air volume over the culture was about 49 c.c. When filled 9 times with air, as in these experiments, it means that 441 c.c. of air supplied the oxygen which was required for a fairly good growth.

The analytical results given in table 8 for tube 1 when recalculated for the total air volume of 441 c.c. show that 71.8 c.c. of CO_2 were produced, and that 90.9 c.c. of O_2 were consumed. These gas volumes are not reduced to standard conditions, but they serve to illustrate the magnitude of the gas exchange. When exact data are wanted, they are obtainable by means of the anaerobe jar, as described in connection with tables 1 and 2.

Paraffin and Rubber Stoppers.—By way of supplementing the results given in table 6, one experiment may be presented showing the comparative behavior of cultures when closed with paraffin or with rubber stoppers, and when glass sealed. The manometric changes in these tubes are given in table 9.

The fall in the negative pressure of tubes 1 and 2 was due to the gradual yielding of the paraffin, the entire plug being slowly forced into the tube. This condition was obviated, in the subsequent tests, by forcing 2 or 3 hot pins through the upper portion of the paraffined plug. It is preferable to insert the pins through the cotton plug before dipping the latter into the molten paraffin. The plugs thus reinforced hold their position perfectly.

On comparing the manometric readings of tube 3, which had a paraffined plug, with those of tubes 8 and 9 (glass sealed), it will be observed that the results were essentially the same. In other words, the paraffin plug did not allow the inward leakage of outside air, neither did it absorb the CO_2 which was formed by the culture.

By contrast, however, tubes 5 and 6, which were securely closed by rubber stoppers treated with glycerol, showed a slow steady rise in the negative pressure. At the end of 28 days, the negative pressure in these tubes was fully 20 mm. higher than in the others. This behavior was just what was expected of tubes sealed with rubber stoppers. Rubber is a solvent for CO_2 , and the manometric difference mentioned implied a loss of about 3% of this gas. The analyses actually showed this to be the case (table 10).

Tubes 1, 2, 10, 4 and 7 were analyzed at the end of 9 to 10 days. They were then evacuated to —300 mm. and refilled with air, the

operation being repeated 10 times. This procedure was repeated at intervals of 5 or 6 days. It will be noted that tubes 1 and 10 were thrice filled with air, while 2, 4 and 7 were thus treated 4 times.

A comparison, at any one period, of the serially aerated tubes will show that the manometric readings of the rubber stoppered tube 4 were higher by 20 mm. or more than the corresponding observations for tubes 1, 2, 10 and 7. This difference, as in the case of the unaerated tubes, is due to the solution of CO_2 by the rubber stopper.

The analytical results obtained in these tests are given in table 10. At the end of the 1st test period, the analyses of the aerated tubes showed the presence of 11-12% of CO_2 , except in the rubber stoppered tube 4, in which only 8.8% was found. At the end of the 2nd test period, the CO_2 content rose to 14-15%, except in tube 4 in which it was 11.4%. Similarly, at the 3rd test period, the CO_2 content rose to 15-16%, while that of tube 4 was only 13.5%. Tube 7 at this point does not lend itself for comparison because of the large content of unconsumed oxygen. The same differences were again brought out at the end of the 4th test period.

The unaerated tubes, which were not analyzed until at the close of the 28th day, showed the same difference as did the aerated tubes at the end of the 1st period. The CO_2 content of tubes 3, 8 and 9 was 11.5-12%, while that of the rubber stoppered tubes was 9 per cent.

It was pointed out in connection with table 8 that the first analyses of a culture tube gave a lower CO_2 content than in the subsequent tests, the difference being due to the partial saturation of the medium by this gas. The very low values obtained with the rubber stoppered tubes in the first analyses were due to the absorption of CO_2 not only by the medium but also by the rubber. In the subsequent tests, the CO_2 value increased somewhat because of the partial saturation of the medium, but it remained consistently lower than in the corresponding tubes because of the continued solvent action of rubber.

The tests given in tables 8 and 10 are conclusive evidence of the fact that the tubercle bacillus can consume, within a few days, practically all of the O_2 contained in a culture tube. For the 20.9% of O_2 removed, it returned more than 17% of CO_2 . It may not be without interest to present, at this point, the results of previous workers.

The first study of the gas exchange of the tubercle bacillus was made by Hesse ⁴ in 1893. He made a series of analyses of the air present

⁴ Ztschr. f. Hyg. u. Infectiouskr., 1893, 15, pp. 17-37.

TABLE 10
RESULTS OF ANALYSES OF AERATED AND UNAERATED CULTURES OF B. TUBERCULOSIS, LISTED IN TABLE 9

Aerated Series			Tube 1			Tube 2			Tube 10			Tube 4			Tube 7		
Closed with.....			Paraffin			Paraffin			Paraffin			Rubber Stopper			Glass Seal		
Test Per.	Dur. Hrs.	Bar. Init.	Corr. Man.	CO ₂	O ₂	Corr. Man.	CO ₂	O ₂	Corr. Man.	CO ₂	O ₂	Corr. Man.	CO ₂	O ₂	Corr. Man.	CO ₂	O ₂
1	204	746	-63.5	12.57	0.11	-82.8	8.84	0.58	-51.7	10.74	4.07
2	252	746	-39	12.48	0.30	12.45	0.58
	64	728	-8.2	15.38	0.52
3	144	756	49.3	14.20	0.36
	212	728	70	11.44	0.27	43.6	15.40	0.64
4	260	756	14.27	0.30	13.50
	112	742	38	16.50	0.29	61.9	32.4	12.51	5.41
4	144	756	44	15.27	0.0	47.7	15.31	0.11	13.29	0.25	44.6
	144	752	41.1	16.11	0.22	13.86	1.88
Unaerated Series			Tube 3			Tube 5			Tube 8			Tube 9			Tube 6		
1	28 d	746	58.5	12.21	0.38	78	9.05	0.13	59	12.33	0.14	78	9.05	0.18	57.9	11.58	0.33
1	28 d	746															
	28 d	746															

in 2 cultures over a period of 152 days. One of the tubes was analyzed 26 times and the other 14 times. The culture medium which he used was blood serum (25 c c.), and it may be inferred from his results that the growth was not rich. In only 2 instances, in which the interval between the analyses was 35 and 36 days, was the O_2 reduced to zero, while the CO_2 rose to 12.5 and 13.4%, which actually were the highest values obtained by him. In more than one-half of the analyses the yield of CO_2 was under 5%.

Assuming that his culture tubes contained 55 c c. of air, the amount of CO_2 produced and of O_2 consumed can be approximately estimated. The values thus obtained are given below under I and II, while III represents those obtained by a similar calculation for tube 1 in table 8.

	I	II	III
C c. CO_2 produced.....	47.5	38.4	71.8
C c. O_2 consumed.....	97.1	68.9	90.9

It will be seen from these figures that the yield of CO_2 in 5 months was considerably less than was calculated for the 1 tube which was aerated only 9 times in 28 days (table 8).

It may be added, at this point, that Moore and Williams¹ made a few analyses of tubes inoculated with the avian tubercle bacillus. These were sealed hermetically or with rubber stoppers. They found only 2.6, 3.0, 5.0, 5.7 and 6.7% of CO_2 , while the O_2 was absent. In one experiment, however, they did obtain 18.7% of CO_2 and no oxygen.

Corper, Gauss and Rensch⁵ likewise determined the CO_2 present in several tubes which were either closed with rubber stoppers or sealed in the flame. They obtained 4.7 to 5.5 per cent. of CO_2 .

INCREASED OXYGEN TENSION

The preliminary studies on the effect of increased oxygen tension were made with culture tubes attached to manometers which had developed good growths as a result of 6 or more refills with atmospheric air. After evacuating such tubes and introducing 30, 40, 60, 80 and 100% O_2 , it was found that the oxygen was consumed and that correspondingly high amounts of CO_2 were produced. An example of this kind is given in table 4, Part I, from which it will be seen that the final analysis showed the presence of 86% of CO_2 . Another instance of this

⁵ Amer. Rev. Tuberculosis, 1921, 5, pp. 562-587.

kind is presented in table 8 in which tests 10 and 11 were made with 20, 30, 40, 50 and 100% O₂.

Having established the fact that a rich culture could dispose of the maximal O₂ tension, it was then desirable to ascertain whether or not the tubercle bacillus would develop in a freshly inoculated tube in varying high tensions of O₂. Experiments made with this object in view demonstrated that it could be grown in all tensions of O₂ up to 100%.

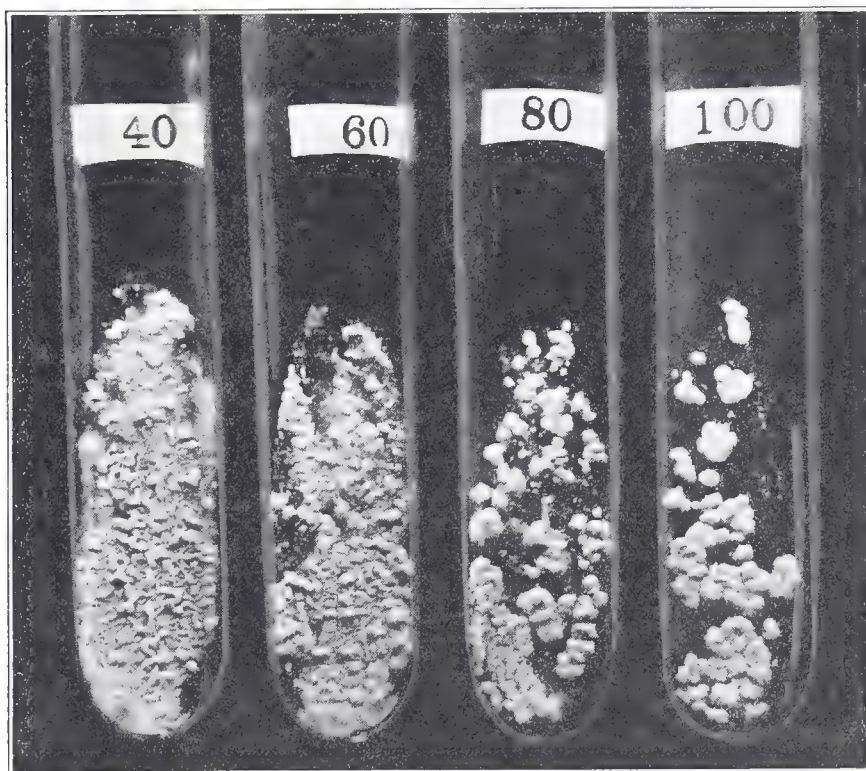


Fig. 6.—Growth of the tubercle bacillus in 40, 60, 80 and 100% O₂, 31 days at 37 C.

The method of procedure was to place, in each of 4 Novy jars, 2 tubes of the slanted agar inoculated with cultures which were 19 to 40 days old. The medium was the usual 1% agar with 5% glycerol, and was slanted over night previous to inoculation. The jars were sealed in the usual way with "Lubriseal," rubber bands and 8 clamps.

The method of filling the jars varied somewhat in the different experiments. One example will suffice to show the general procedure followed. Thus, jar 1 was evacuated to -170 mm., after which O₂ was admitted until the vertical manometer showed normal or zero pressure. Similarly, jar 2 was evacuated to -350 mm., and then O₂ was let in to the zero point. Through the other 2 jars, O₂ was passed for 2 hours in order to displace completely

the contained air. Jar 3 was then evacuated to -175 mm., and air was admitted until the pressure reached zero. Jar 4 presumably had the full O_2 content which, however, was less than 100% because the gas in the tank was never strictly pure.

After filling, the jars were placed in the hot-room over night, and then the actual percentage composition was determined by analysis. They were then kept at $37^\circ C.$ for 4 weeks or longer.

Repeated experiments made under these conditions gave the same results, provided no mercury was introduced into the jar while drawing the initial sample. The effect of the presence of mercury will be discussed later. Fig. 6 shows the appearance of the cultures grown for 31 days in 40, 60, 80 and 100% oxygen. Actually, the initial analysis showed the presence of 39.4, 56.7, 81.6, and 93.1% of oxygen.

It is noteworthy that at times growth was recognizable in the jars with 40 and 60% O_2 as early as the 3rd day. It was especially observable on the surface of the water of condensation, forming a pellicle which rapidly spread. By the 7th day, a good growth covered the surface of the agar; and after this period it rapidly increased. On the 14th day, it was very rich and better than that in "holed" sealing-wax tubes or ordinary controls. As a rule, there is little difference between the tubes in the 40 and 60% O_2 atmospheres. In the latter, the growth may show some retardation over the former, but in the end they are alike.

On the other hand, the growth in the jar with 80% O_2 was distinctly retarded. While it did not completely cover the surface of the agar, it was thicker and showed but slight tendency to form wrinkles. The growth in the water of condensation was distinctly different from that in 40% O_2 . In the latter, it spread over the surface and crept up over the glass, while the liquid remained clear. In 80% O_2 , the growth did not form the usual pellicle, or spread over the glass, but rather descended into the liquid forming streamers or stalactites.

In the 100% O_2 jar, the characteristics of the culture were like those in the 80% concentration but more pronounced. The surface growth consisted almost entirely of isolated, white, thick, moist, smooth, convex colonies (3-5 mm.). It would seem as if relatively few organisms were able to grow under this extreme condition. The growth was associated with the size of the particle deposited on the medium at the time of inoculation. The appearance of these colonies was unlike that of the ordinary culture. Equally striking was the appearance of the growth in the water of condensation. At first, it was stalactitic, but later it

became so abundant as to fill the liquid. The growth was carried through 3 successive generations in 100% O₂.

TABLE 11

RESPIRATION OF *B. TUBERCULOSIS* IN HIGH O₂ TENSION, 2 TUBES, GLYCEROL AGAR, 55 DAYS, 37 C.

Experiment No.	1	2	3	4
Planned percentage O ₂	40	60	80	100
Net gas volume.....	1864	2170	2072	1697
Initial analyses				
CO ₂	0.359	0.361	0.0	0.277
O ₂	37.914	56.064	76.618	96.600
N ₂	61.727	43.575	23.382	3.123
Final analyses				
CO ₂	23.077	16.085	14.788	13.510
O ₂	12.751	38.971	60.985	83.581
N ₂	64.172	44.944	24.227	2.909*
Corr. analyses				
CO ₂	22.198	15.595	14.272
O ₂	12.265	37.784	58.856
N ₂	61.727	43.575	23.382
	96.190	96.954	96.510
Unreduced				
C c. CO ₂ produced.....	407.1	330.6	295.7
C c. O ₂ consumed.....	478.1	396.7	368.0
Real resp. quot.	0.851	0.833	0.803
Growth.....	10+	8+	6+	3+

* When nitrogen is present in small amount any error in analysis is greatly exaggerated in the nitrogen factor, which as a result is either very low or very high. Hence the "corrected" analysis in such case is of no value. In this instance the calculated respiratory quotient would be 2.07 which is obviously impossible.

In table 11 are presented the analytical results obtained in one experiment with the 4 concentrations of O₂ mentioned. The duration of this experiment was 55 days. The table shows that considerable gas exchange occurred, but with a distinct decrease as the O₂ concentration increased. This, of course, could be expected from the appearance of the mass growth in the tubes. The number of c c. of CO₂ produced in 55 days can be compared with the values given in table 5 for cultures grown 27 days in air. In the 40% O₂ jar the yield per tube was 203 c c. as against a maximum of 151 c c. in table 5. Similarly, the O₂ consumed in the former was 239 c c. per tube as compared with 182 c c. in the latter. These analyses bore out the objective findings, namely, that better growths were obtained in 40% O₂ than in ordinary air (20.93%).

One experiment, similar to the foregoing, was made with 1% agar containing 2% of glucose. In 40% O₂, a rich growth was obtained

in less than 2 weeks. In 60% O_2 , only about 8 colonies developed on the slant, but the water of condensation had a rich growth. Several very minute pinheads of mercury were found in this jar. The same result was obtained in the jar with 97.5% of O_2 where several small globules of mercury were present. In the jar with 80% O_2 , more mercury was present than in the others, and as a result no growth occurred on the slant although it was present in the water of condensation. The accidental presence of Hg in a jar prevents growth, as will be shown later.

The growth of the tubercle bacillus in high O_2 tension was studied by Moore and Williams.¹ They arrived at the conclusion that the tubercle bacillus was killed when the concentration of O_2 exceeded 80%. Adams,⁶ using the same methods, secured no growth in 90%, but he obtained in 85% O_2 , in 6 weeks, raised, discrete, dark brown masses. He therefore concluded that the high O_2 concentrations were inhibitive and not bactericidal.

These results are somewhat at variance with those obtained in this study. We have had no difficulty in obtaining growths in 98% O_2 in from 2 to 3 weeks. Moreover, the growth was pure white although in raised, discrete masses. The failure of Moore and Williams to obtain growth was due, in part, to the presence of soda-lime which was placed in the bell-jar "to absorb any CO_2 set free." It will be shown later that the dehydrating action of KOH solution is sufficient to inhibit the growth of the tubercle bacillus. Soda-lime can be expected to be similarly inhibitive. Although Adams makes no mention of having used soda-lime, it is reasonable to believe that he did so, since his work was done in Moore's laboratory.

Another source of error in the work of Moore and of Adams was in the use of a mercury seal around the base of the bell-jar. The fact that Adams described the growths which he obtained as dark brown in color may be considered as evidence of leakage of mercury into the bell-jar. It will be shown later that the tension of mercury is sufficient to inhibit the growth of the tubercle bacillus, and that if growth does occur it will be dirty grayish-black, due to the formation of mercuric sulphide. In the absence of mercury, the culture in 100% O_2 remains pure white.

DECREASED OXYGEN TENSION

It has been pointed out that the tubercle bacillus, in order to produce a rich growth, must be provided with about 100 c.c. of O_2 or about

⁶ Biochem. Jour., 1912, 6, pp. 297-314.

500 c.c. of air. Any conclusion which is based on a limited quantity of air, as for example that which is present in a culture tube, is necessarily erroneous. Similar errors have been perpetrated in tests made to determine the lower limits of O_2 concentration which will permit growth.

It is obvious from what has been said that the question of the effect of decreased oxygen tension cannot be positively answered unless the absolute amount of O_2 which is present is sufficient to meet the requirements of the organism. With this fact in mind, and since previous experiments had shown that the tubercle bacillus grew well in 10% of O_2 , it was planned to determine the effect of O_2 concentrations corresponding to 6, 3, 1 and 0.5%.

The experiments were carried out by making use of containers which progressively increased in size in order to supply the required absolute amount of oxygen. A jar of 2,000 c.c. capacity, with an atmosphere containing 6% O_2 , should provide 120 c.c. of O_2 for the organism.

The tall form of the Novy jar (Part I) which has an air capacity of about 3,300 c.c. with an atmosphere containing 3% of O_2 would provide an actual stock of 99 c.c. of oxygen.

As a container for the atmosphere which was to have 1% of O_2 , a bottle of 7.7 liter capacity was utilized. This therefore would actually hold 77 c.c. of O_2 , which should suffice to give a good growth.

For the experiment with 0.5% oxygen, it was necessary to provide a still larger bottle, one with a capacity of 20 liters, thus supplying 100 c.c. of O_2 .

Into each of these containers, 2 c.c. of water were introduced to supply the needed aqueous tension; likewise, one freshly inoculated culture tube containing 10 c.c. of the medium. The medium contained, as usual, 1% agar and 5% glycerol. The tubes were loosely plugged to favor gaseous diffusion. The jars were sealed in the same manner as those employed in the work with high tensions of oxygen. Each bottle was closed with a rubber stopper (No. 25) treated with glycerol and provided with a tail-cock (fig. 13, Part I). In the case of the 20 l. bottle, which on account of its size had to be kept in an inclined position, the arm of the tail-cock was bent to a vertical position.

The smaller jar, no. 1, was then evacuated to —550 mm. and washed nitrogen admitted until the pressure dropped to zero. The content was analyzed after 2 hours, and 6.7% of O_2 were present. As this was higher than was desired, the jar was evacuated to —15 mm., and nitrogen was admitted. Analysis now showed 5.31% of O_2 .

The tall jar, no. 2, was evacuated to —650 mm., and pure nitrogen was admitted until the pressure fell to the zero point. Two hours later, analysis gave 2.48% of O_2 . In order to bring this up to 3%, the jar was evacuated to —15 mm., and air admitted. Analysis now showed 2.9% of oxygen.

The smaller bottle, no. 3, was evacuated to —400 mm., and refilled with pure nitrogen. This operation was repeated 5 times. Since the analysis, after 2 hours, showed only 0.54% of O_2 , the bottle was evacuated to —15 mm., and air was admitted. Analyzed at the end of 2 hours, it now contained 1% of oxygen.

The large bottle, no. 4, was similarly evacuated 5 times to -400 mm., pure nitrogen being admitted after each evacuation. The gas in the bottle was analyzed 4 hours later and was found to contain 0.85% of O_2 . The bottle was therefore again evacuated to -400 mm. and pure nitrogen admitted as before. When analyzed some hours later, 0.51% of O_2 was found to be present.

The containers were then set aside at $37^\circ C.$, the large bottle being placed in an inclined position. As controls, 4 additional tubes of the medium were inoculated, closed with sealing-wax and holed in the usual way.

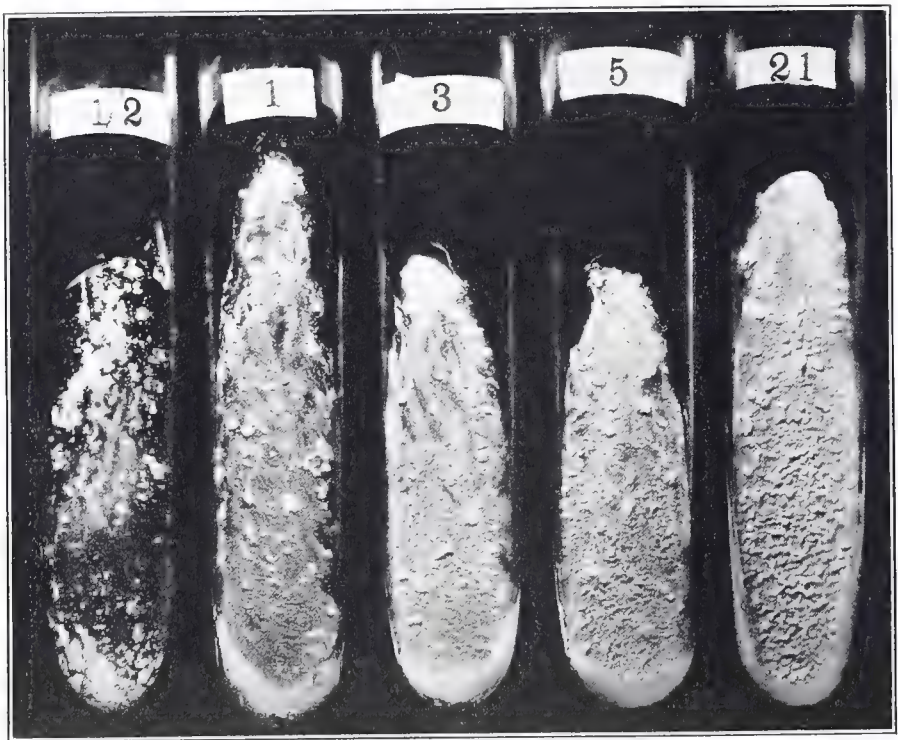


Fig. 7.—Growth of the tubercle bacillus in 0.5, 1, 3, 5 and 21% O_2 , 42 days at $37^\circ C.$, except 0.5, which was 49 days.

On the 5th day, the relative growths in the controls and in the O_2 containers (from 1 to 4) were, respectively, 5 +, 3 +, 2 +, 1 + and 0. On the 14th day, they were scored as 10 +, 10 +, 6 +, 3 + and 1 +. In other words, the growth in the jar with 6% O_2 was about as heavy as those in the control tubes (10 +). The other tubes showed a progressive decrease in the mass of the growth corresponding to the decreasing percentage of oxygen present in the respective containers.

It was evident that the rapidity of the utilization of O_2 , and hence of growth, depended on the concentration. This was in accord with the previous observations that in 40% O_2 the growth was more rapid

than in ordinary air. The limit of favoring action in those tests appeared to be at about 50% above which retardation was observable.

Since the absolute amounts of O_2 present were 119, 95, 76.6 and 99 c.c. it was to be expected that when all of the O_2 had been utilized, the final growths would show a corresponding difference in the 4 tubes. The appearance of the tubes at this time, when all of the O_2 had been consumed, is shown in fig. 7.

This illustration is conclusive evidence of the fact that the tubercle bacillus can grow in the culture tube under any decrease in the O_2 tension. The only limit imposed is the size of the container which, for reasons stated, must be of such capacity as to provide approximately 100 c.c. of O_2 . After all, the fact demonstrated merely parallels the conditions in the body in which the organism grows under a constant, though greatly diminished, O_2 tension.

It will be seen from Table 5 that a single culture of the tubercle bacillus consumed from 153 to 182 c.c. of O_2 in 26 to 27 days. In view of this fact, it might be expected that all of the O_2 present in these experiments, being less in amount than that mentioned, would be consumed in the same period of time. This, however, was not the case, for the reason that the low O_2 tension that was present at the start, and its subsequent further decrease as growth developed, made it impossible for the organism to multiply at the same rate that it would have done in the presence of an excess of O_2 .

The low tension resulted in a lessened multiplication and a slower removal of the residual O_2 . Thus, tubes 1 and 2 required 5 weeks, while tube 3 took 6 weeks to effect complete removal of the O_2 . Tube 4, which was in an atmosphere containing only 0.5% O_2 , at the end of 7 weeks had reduced the O_2 tension to zero. On reference to table 12, it will be seen that at the end of 4 weeks the O_2 consumption in the 4 containers was 91.9, 86, 79 and 30.4%, respectively.

The striking fact revealed by figure 7 is that the growth decreased progressively as the O_2 tension was lowered. Thus, the growth in the bottle with only 0.5% O_2 was greatly inferior to that in the jar which contained 3% O_2 ; and yet, the absolute amount of O_2 consumed was practically the same, being 99 c.c. in the former and 95 c.c. in the latter. Again, the growth which developed in the bottle with 0.5% O_2 and which consumed 99 c.c. was less in amount than that which formed with only 76 c.c. of O_2 and under the initial tension of 1% O_2 .

It is evident that the mass action of O_2 is the factor to be considered here. The rate of the respiratory process is clearly lessened as the tension of the O_2 is decreased. It may be assumed that the respiratory quotient is a constant which is independent of the O_2 pressure and, if so, the total energy liberated by a given amount of O_2 , *e. g.*, 100 c.c., should be the same regardless of the existing tension. On the other hand, if the slower rate of energy production, under decreased O_2 tension, be attended with a considerable loss of energy by dissipation the result would be a growth mass smaller than that which would form under higher tensions.

TABLE 12
RESPIRATION OF *B. TUBERCULOSIS* IN LOW O_2 TENSION, 1 TUBE, GLYCEROL AGAR, 37 C.

Experiment No.	1		2		3		4	
Planned percentage O_2	6		3		1		0.5	
Net gas volume.....	2,243		2,277		7,665		19,475	
C c. O_2 present.....	119.3		95.0		76.6		99.3	
Analyses	CO ₂	O ₂	CO ₂	O ₂	CO ₂	O ₂	CO ₂	O ₂
Initial.....	0	5.32	6	2.90	0	1.0	0	0.51
14 days.....	0.043	0.426
28 days.....	4.06	0.43	2.34	0.41	0.63	0.21	0.078	0.355
35 days.....	4.576	0.0	2.468	0.0	0.768	0.032	0.14	0.27
42 days.....	4.55	0.0	2.58	0.0	0.803	0.0
49 days.....	0.359	0.0
Percentage of initial O_2 consumed in 28 days.....	91.9		86		79		30.4	
Real resp. quot. 42 days.....	0.848		0.886		0.801†		0.702†	
Growth, 42 days *.....	8+		7+		5+		4+	

* The growth in a holed, sealing-wax control culture was 10+.

† This value is low probably because some CO_2 was taken up by the rubber stopper.

Mention may be made at this place of an experiment in which one freshly inoculated tube of glycerol agar was placed in a jar having a capacity of 2,154 c.c. It was intended to fill the jar with pure N_2 , but on analysis it was found to contain 0.19% of O_2 and no CO_2 . When analyzed again at the end of 4 weeks, the O_2 had entirely disappeared and 0.14% CO_2 was present. The experiment showed that the organism could utilize a concentration of O_2 which was as low as 0.19%. It would have necessitated the use of a container of about 40 liter capacity to obtain a visible growth. With the jar used, there was no evidence of growth showing that the organism was unable to multiply as an anaerobe in an atmosphere of almost pure nitrogen. That the culture was viable

at the end of the 28-day period was demonstrated when the tube was removed from the jar, closed with sealing-wax and holed. Within a week, a good growth appeared in the tube.

It is hardly necessary to point out that these experiments are of considerable importance. They demonstrate, for the first time, the relation of low O_2 tensions to the growth of the tubercle bacillus. It is precisely under conditions such as these that the organism finds itself when in the body. The tension of O_2 in the tissues of the body is unknown. It is much less than that in the arterial or venous blood. The tension of oxygen in abdominal air, about 45 mm. Hg, was considered by Haggard and Henderson⁷ as corresponding probably to that of the tissues. In the absence of direct determinations, it must be assumed that this value is too high. It is not unlikely that the O_2 demand of the cells, constituting a tissue mass, is such as to reduce the O_2 tension to a few mm. of Hg.

The fact that anaerobic bacilli and spirochetes do grow in the tissues is evidence of such low O_2 tension. It is also indicated by the well-known fact that the tissues decolor or reduce many dyes when these are injected intravenously (Ehrlich, 1885).

The chronicity of tuberculosis points to a slow growth of the tubercle bacillus which, in turn, is indicative of a very low O_2 tension. It must multiply much slower in the body than it does in the culture tube in the presence of air where the O_2 tension is about 150 mm. of Hg. It would seem that within a mass of pus, or of caseating matter, the O_2 tension must be reduced almost to the zero point. The organism cannot grow in the total absence of oxygen but it can multiply, though slowly, in a tension which is as low as 3.5 mm., as demonstrated in exper. 4 of this series. This O_2 tension is such that water at 0 degree, 760 mm., would take up 0.02 volume % of O_2 , and, as shown in Part IV, this amount would permit the growth of some anaerobes in liquid or solid mediums. In other words, the tubercle bacillus can grow in overhead concentrations of O_2 which are low enough to permit multiplication of obligative anaerobes.

The question may be raised as to whether or not the beneficial results obtained in the "rest cure" in tuberculosis are associated with a lowering of the O_2 tension in the tissues thereby lessening the multiplication rate of the organism. Similarly, the spontaneous cure in this disease may find its real explanation in the complete removal of O_2 from the immedi-

⁷ Jour. Biol. Chem., 1919, 38, p. 78.

ate vicinity of the organism, thus causing cessation of its growth and eventually its death. Again, the undoubted effect of a rich diet may be associated, in large part, with the reducing power of the tissue-fat. An increased deposition of fat would tend to lower the O_2 tension in the tissues and would thus lower the rate of growth of the germ. Obviously, the study of the disease from the standpoint of lessened O_2 tension is indicated by this experimental work.

INCREASED CO_2 TENSION

It was shown earlier in this paper that the culture on glycerol agar consumed all of the oxygen in the air and returned more than 17% of CO_2 . Similarly, on glucose agar, the organism produced 20% of CO_2 . In tubes, which were filled with concentrations of O_2 higher than that in air, there was a corresponding increase in the yield of CO_2 . In one experiment of this kind, summarized in table 4, Part I, the return of CO_2 amounted to 86% (uncorrected).

It was evident from these facts that the tubercle bacillus was actually capable of growing in relatively high concentrations of CO_2 . However, it was desirable to establish this fact by developing freshly inoculated tubes in atmospheres which, from the start, contained varying percentages of CO_2 . Preliminary trials showed that 10% CO_2 had absolutely no inhibiting action on the growth of the organism. Consequently, it was decided to test out atmospheres which contained 30, 40, 50, 60, 80 and 90% of CO_2 .

It has been repeatedly emphasized that in order to obtain a successful culture of the tubercle bacillus an adequate supply of oxygen must be insured. In sealed, ordinary culture tubes, the amount of air is wholly insufficient to promote growth (figs. 2-5). Good growth can be expected only when at least 75 to 100 c c. of O_2 are available. Hence, the necessity of resorting to the use of the jar method of cultivation.

In the first experiment of this kind, 2 freshly inoculated tubes of glycerol agar were placed in each of 4 jars. These were then sealed in the usual way. Jar 1 was now evacuated to -225 mm. and then CO_2 was admitted to zero pressure. Jar 2 was pumped out to -300 mm., and CO_2 allowed to run in to the zero point. Jar 3 was evacuated to -375 mm. and then refilled with CO_2 . Jar 4 was evacuated to -450, after which CO_2 was admitted. The jars thus treated were then placed at 37 C. They were analyzed a few hours later, and again on the 31st and on the 59th day. The results are given in table 13.

It will be noted that on the 59th day practically all of the O_2 was removed from jars 1, 2 and 3. In jar 4, the O_2 was reduced to about

one-third, since the growth in 60% CO₂ was less in amount than in the other concentrations. The uncorrected increase in CO₂ in this jar was about 6%, whereas in the other 3 jars it approximated 12%.

A good demonstration of the growth obtained under these conditions is given in fig. 8. The growth in 30, 40 and 50% CO₂ was well developed on the 31st day; whereas in the 60% jar it was doubtful. After that day, however, the growth made its appearance. It showed no tendency

TABLE 13
RESPIRATION OF *B. TUBERCULOSIS* IN HIGH CO₂ TENSION, 2 TUBES, GLYCEROL AGAR,
59 DAYS, 37 C.

Experiment No.	1	2	3	4
Planned percentage CO ₂	30	40	50	60
Net gas volume.....	2066	1861	2170	2072
Initial analyses				
CO ₂	29.85	38.02	49.14	61.75
O ₂	16.12	13.59	11.03	8.98
N ₂	54.03	48.39	39.83	29.27
Final analyses				
CO ₂	42.46	50.47	59.17	67.54
O ₂	0.54	0.34	0.34	2.88
N ₂	57.00	49.19	40.49	29.58
Corr. analyses				
CO ₂	40.248	49.649	58.206	66.831
O ₂	0.512	0.334	0.334	2.849
N ₂	54.030	48.390	39.83	29.270
	94.790	98.373	98.370	98.950
Unreduced				
C c. CO ₂ produced.....	214.8	216.7	196.7	105.3
C c. O ₂ consumed.....	322.5	247.1	232.1	127.0
Real resp. quot.	0.666*	0.877	0.848	0.828
Growth.....	10+	10+	10+	3+

* Evident error in the fractional analysis of the initial gas.

to spread, but formed raised, discrete, pure white colonies. Attention should be called to the heavy surface growth on the water of condensation in the tubes from jars 1, 2 and 3.

It may be added that a subculture made from the growth in jar 4 and placed in another jar with 60% CO₂ gave as heavy a growth in 22 days, as did a like subculture in a "holed" sealing-wax tube. The culture in 60% CO₂ was therefore viable even at the end of 59 days. Without doubt, it could be carried through any number of subcultures in the same CO₂ concentration.

These experiments were repeated a number of times with like results. In 2 instances, the concentrations of CO₂ were changed to 40, 60, 80 and 90%. The O₂ content was raised to 20% in the first 3 jars, and to 10% in the fourth.

Good growths were obtained in each of these concentrations in 57 days. The growth in 90% CO_2 was slow to develop and was not as rich as in tubes with 90% O_2 . It took on the form of isolated, raised blisters. It was carried through 3 subcultures in this same concentration of CO_2 .

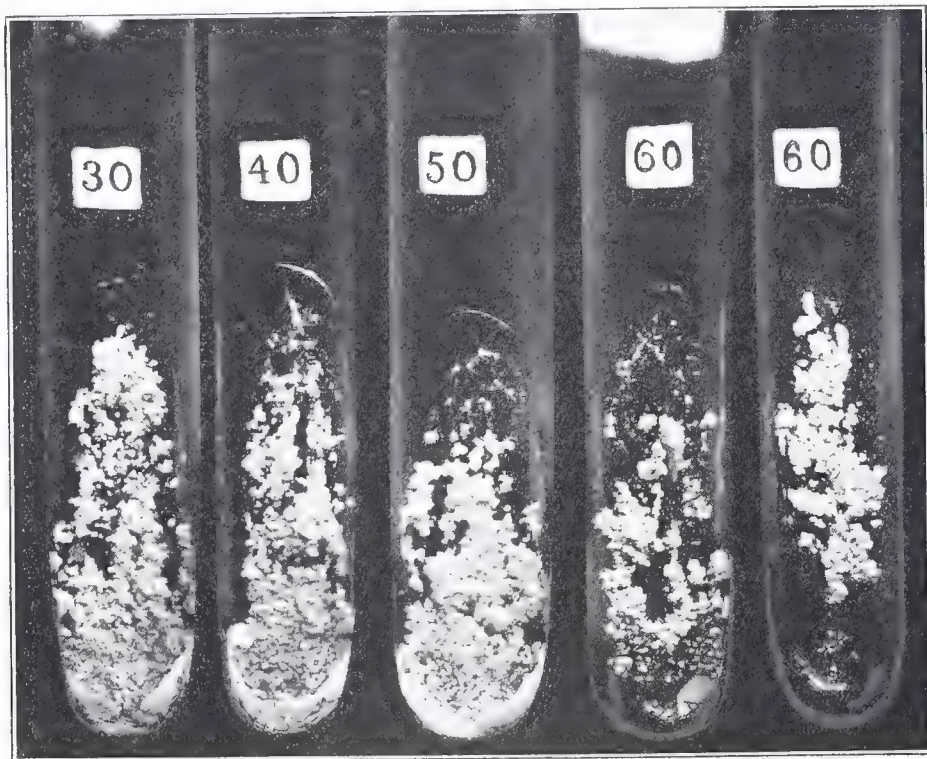


Fig. 8.—Growth of the tubercle bacillus in 30, 40, 50 and 60% of CO_2 , 60 days at 37 C.

It is evident from the foregoing that the tubercle bacillus can grow very well in concentrations of CO_2 up to 60%. It will also grow, though more slowly, in concentrations of 80 and even 90% of CO_2 . The results of Corper⁵ and his co-workers which indicated that a concentration of approximately 5.5% of CO_2 inhibited the growth of the tubercle bacillus and that 15% was tuberculocidal, are quite at variance with those obtained in this study.

DECREASED CO_2 TENSION

As mentioned above, Moore and Williams¹ (1909) obtained no growth of the tubercle bacillus in atmospheres with high O_2 tensions.

Looking only at the question of O_2 tension, they entirely lost sight of the possible effect of the soda-lime which was present. They recognized that the soda-lime was effective as regards the removal of CO_2 since their analysis showed an entire absence of this gas. Instead of ascribing the failure to secure growths to the high O_2 tension, with equal propriety, they might have considered it as due to the absence of CO_2 . This was actually what Wherry and Ervin⁸ (1918) did when they found that this organism failed to grow in the presence of alkali. The view that tubercle bacilli will not grow in a carbon dioxide-free atmosphere was likewise emphasized by Corper⁵ and his co-workers.

As this idea seemed to be of considerable biologic interest, it was subjected to a thorough inquiry. Probably no phase of this study received as much attention as did this question. It was not a matter which could be solved readily and beyond doubt.

Preliminary work was done with tubes having the *h*—and 3-prong form (figs. 1 *B* and 1 *C*, Part I). These tubes received the medium in the main arm, and 5 c.c. of 10% KOH in the side-arm. After inoculation, they were closed with sealing-wax or with rubber stoppers treated with glycerol. The latter were usually provided with a tail-cock (fig. 13, Part I) which in some tests was left open; in others it was closed. Similarly, the tubes with sealing-wax were either completely sealed or were provided with finely drawn capillary tubes which were passed through the wax and the cotton plug.

It was found that, after 28 days, a growth in the form of thick isolated colonies slowly developed, especially in the tubes which were provided with capillaries or open cocks. In the tubes thus kept open to provide an abundance of O_2 , the growth was somewhat richer than in the closed tubes, but not as rich as in the corresponding controls.

That respiration was slowly going on in the closed tubes was revealed by analysis. At the end of 14 days, such tubes contained no CO_2 and only from 7-14% of O_2 . This was considerably more than was present in control tubes which had water in the side-arm in place of the alkali. Hence, the consumption of O_2 , though greatly retarded, was nevertheless evident.

After the analysis, the tubes were refilled with pure air, and they were again analyzed at the end of 7 days. This time the O_2 content was found to be less than in the previous analyses which were made at the close of 14 days. A second refill gave an even more marked reduction of O_2 , showing that the organism was multiplying, though slowly, in an apparently CO_2 -free atmosphere.

It was evident from these experiments that some growth did occur in the presence of KOH. Although analysis showed the absence of CO_2 , it could nevertheless be supposed that some CO_2 was present within the small growing mass and that in this way some stimulus was given to the organism. A definite conclusion, therefore, could not be reached by this method of experimentation.

Accordingly, a wholly different procedure was resorted to. Conceivably, a satisfactory solution could be obtained by a rapid aeration of freshly

⁸ Jour. Infect. Dis., 1918, 22, pp. 194-197.

inoculated tubes, some with CO₂-free air, and some with air containing CO₂.

For this purpose compressed air was used. It was rendered CO₂-free by passage through a high column of soda-lime (2 x 60 cm.). It was then supersaturated with moisture. This could not be affected by any ordinary arrangement of wash bottles but was readily accomplished by sending the air through a slightly shaven, sterile Berkefeld bougie (2.5 x 18 cm.) which was attached to a Novy⁹ filtering cylinder. The latter contained about 1,500 c.c. of sterile distilled water. The CO₂-free compressed air, under a pressure of 12 lbs., was forced outward through the wall of the bougie in a stream of bubbles, which, passing through the water at 38 C., became supersaturated.

The saturated air was led from the top of the glass cylinder to a glass Y, which divided the stream of air. The current from one arm was passed through some water in a test-tube on foot, provided with a side-arm outlet. By this means, the rate of flow could be observed and controlled. The outlet in turn was connected to a Pasteur pipet, which was passed through a doubly perforated rubber stopper. When inserted into the culture tube, the tip of the pipet reached the bottom of the tube just above the inoculated medium. The outflow from the tube was carried through a small bottle containing some liquid paraffin. The object of the latter was to arrest any tubercle bacilli which might have been picked up by the rapid air current.

The other arm from the Y was connected, by means of a doubly perforated stopper, to another test-tube on foot. The second perforation carried the delivery tube from a CO₂ tank. The air and the CO₂ bubbled through the water in this tube and the resulting mixture was then passed over the culture in a tube, in the way mentioned.

By this arrangement, it was hoped, that it would be possible to remove all of the CO₂ from tube 1 as fast as it was formed by the culture. If the CO₂ was indispensable, then little or no growth should result. On the other hand, in tube 2, on account of the presence of CO₂ in the air, a good growth should develop, notwithstanding the fact that it was being aerated at the same speed as tube 1.

The entire apparatus, though seemingly complicated, worked quite satisfactorily. In 11 experiments which were run continuously over a period of 6 months, contamination of the cultures rarely occurred. There was some trouble either with desiccation or with flooding, by condensation of moisture.

The rate of flow through the culture tube was varied in the different experiments, but usually was maintained at 300-400 c.c. per minute. The outflow air from tube 1 whether slow or fast when sampled by means of a Bailey bottle was found to contain so little CO₂ that the analysis of 10 c.c. almost invariably showed nothing. The organisms in this tube were therefore constantly in a CO₂-free atmosphere. The CO₂ content of the air which was passed through tube 2 was varied, in the different experiments, from 1 to 12% and even more, though it was usually kept at about 3%.

The inoculation of the medium in these tubes was not made by means of a spatula, as was usually done. Instead, the culture was transferred to sterile broth in a test-glass and rubbed up so as to form a fine suspension. After allowing about 5 minutes for the larger particles to settle, the slightly cloudy, supernatant liquid was transferred by means of a Pasteur pipet to the medium, and then was carefully spread all over the surface. This method of inoculation was intended to avoid all possibility of localized respiration within a small mass of the inoculum.

⁹ Centralbl. f. Bakteriöl., I, 1897, 22, p. 337; 1903, 35, p. 126. Laboratory Work in Bacteriology, 1899, Ann Arbor, Wahr, p. 471.

The results in these experiments varied considerably with the physical conditions within the tubes. They were not as convincing as had been expected. In 3 or 4 of the trials, the best cultures were those exposed to the stream of CO_2 . In 4 of the tests, the CO_2 -free atmosphere furnished the best cultures, while in the others the results were about alike. The fact that the CO_2 -free atmosphere gave any growth at all, and that on the whole it gave fully as good results, if not better, than the one which contained CO_2 , forced the conclusion that a CO_2 atmosphere was not essential to the growth of the tubercle bacillus. However, the possibility of a localized production of CO_2 within small masses or aggregates was not entirely set aside.

In view of these results, another line of experimentation was decided on. It was desirable to remove, as fast as possible, the CO_2 which was being formed in a minute mass of the inoculum. With this object in view, 20 c.c. of glycerol agar (2%) were poured into a Petri dish. When thoroughly set, the surface of the agar was inoculated by means of a spatula, and the material was spread as well as possible. The dish was then inverted over another bottom dish of like diameter, in which 10 c.c. of 5 or 10% KOH were placed. The two halves were secured together by means of 3 short pieces of adhesive tape. Three such plates with alkali were placed on the bottom of an anaerobe jar. On top of these, as a control, a similar plate was placed, inverted however, over 10 c.c. of sterile water. The jar was then sealed and set aside at 37 C. The use of the jar removed all question of an adequate supply of oxygen.

At the end of 56 days, the jars were opened and the plates examined. The results seemed to be perfectly conclusive of the need of CO_2 for the growth of the organism. The plate, which was inverted over water, was covered with a rich growth. The 3 plates which were inverted over 10% KOH showed practically no growth, or at most isolated colonies which were only 1 or 2 mm. in diameter. By contrast, however, the 3 plates, over 5% KOH, were covered with larger colonies (3-5 mm.). The alkali in the latter set showed a deposit of KHCO_3 , and it appeared as though the better growth in the plates were due to neutralization of the KOH.

The inference that KOH inhibited the growth of the organism by the mere removal of CO_2 was impaired by the fact that an appreciable desiccation had taken place. It was possible that the real factor in all these experiments was the removal of moisture by the hygroscopic alkali. As stated above, the plates which were inverted over water, although confined in the same jar with 3 plates of alkali, gave excellent growths. Obviously, they were not as CO_2 -free as the other plates, but at the same time the concentration of CO_2 could not have been great. The rich growth could properly be ascribed to the presence of moisture.

In another experiment, such as that given above, the jar was opened at the end of 25 days. The water plate again showed an excellent growth of colonies (2-10 mm.), whereas the plates which were inverted

over the alkali had mere pin-point colonies. To show that it was the medium which was responsible for the poor growth, the alkali bottoms were now replaced with like dishes containing water and then the plates were returned to the jar. When examined 49 days later, it was found that the colonies had increased in size, up to 2-5 mm., but the growth could not be compared with that on plates kept over water and unexposed to the desiccating action of alkali. This test therefore showed that the medium had become altered as a result of exposure to the alkali.

The change was therefore either due to desiccation or possibly to some unknown effect of alkali tension. The latter possibility was tested by inverting agar plates, colored with phenol red, over the alkali bottoms and placing these in jars at 37 C. Such plates showed no change in the P_H value after 2 months' exposure to alkali, and consequently the alkali tension was a negligible factor.

Three series of experiments were now undertaken to counteract the dehydrating action of the alkali. In these tests, the bottom of the jar was covered with water to a depth of about 1.5 cm. In order to have a free interchange of air, a glass triangle was placed on the rim of the KOH dish, and then the inverted agar plate was set on top. Each pair of dishes was secured in position by 3 strips of surgical tape. They were then stacked on top of a glass tripod within the jar. This was then closed as usual, and connected with the stream of supersaturated air which was obtained by means already described.

In one experiment in which the air was sent through the jars at the rate of 400 c.c. per minute, analyses of the outgoing air were made at 5 intervals during the period of 28 days. The CO_2 content was found to be 0.02, 0.01, 0.0, 0.05 and 0.0%.

The appearance of the colonies on these inverted plates was striking. Instead of spreading in the usual way, the colonies grew downward, like stalactites. The columnar growth appeared to be from 1 to 4 mm. high. It was noticeable as early as the 3rd day. The jars in this experiment were opened on the 28th day. As usual, the top or water plates had the richest growth, but the plates over the alkali showed much better development than did those over alkali in an unaerated jar. Analysis of the air in the latter jar gave 0.13, 0.19, 0.26, 0.24 and 0.37% CO_2 . It therefore contained considerably more CO_2 than did the aerated jars, and yet the growth on the plates was greatly inferior, without doubt due to the drier atmosphere.

Figures 9 and 10 show the appearance of the growths in 28 days on 2 plates which were contained in the same aerated jar. The culture



Figs. 9 and 10.—Growth of the tubercle bacillus on inverted plates, the former over water, the latter over KOH, in a jar through which moist air was passed, at a high speed. The poorer growth in no. 10 was due to greater desiccation.

dish in the former was inverted over water and served as a control, while the latter was over 10 c.c. of 10% KOH. The control plate was not as rich as it usually was because of evident desiccation due to the high speed at which aeration was carried on. The volume of the agar in this plate at the close of the experiment was 9 c.c., while that of the plate over KOH was 5 c.c. The contrast between the two plates is therefore due to unequal desiccation and not to the absence of CO_2 .

In the experiment just mentioned, the aeration was carried on at the rate of 400 c.c. per minute. Though the air was presumably supersaturated, dehydration took place because of this speed. The effect of a lower speed of aeration is brought out in figs. 11 and 12. The 2 plates were contained in the same jar through which the moist air was passed at the rate of 75 c.c. per minute. The plate in fig. 11 was inverted over water and corresponds to fig. 9, while that in fig. 12 was over KOH. The analyses of the air content, at intervals, showed the presence of 0.27, 0.13 and 0.17% CO_2 . The growths on these plates was much better than those shown in figs. 9 and 10. It was, however, not because the percentage of CO_2 was higher, but because the lower speed of aeration produced less desiccation.

It may be mentioned that the water and alkali below the inverted plates became cloudy, and showed a film on the surface. This consisted of aggregates of granules or needles which, to some extent, were rodlike and might have been mistaken for the tubercle bacillus. It was more likely that the cloudiness was due to a heavy volatile product of the organism.

The growth on the plates over alkali was not as good as had been expected, and the reason for this was clear. The plates as stated showed marked evidence of desiccation. To ascertain the extent to which this had gone, the agar in the plates was liquefied, and the resulting volume was measured. In one such test, under rapid aeration, the plate over water gave 9 c.c. while those over alkali gave 5.0 to 6.5 c.c., instead of 20 c.c., the amount originally present. Obviously, under these conditions, the growth of the organism was greatly impeded. It is evident that in experiments of this kind, the plates or tubes which are exposed to the action of alkali should be weighed before and at the close of the test, to determine the extent of the desiccation.

These experiments all go to show the difficulty of counteracting the dehydrating action of alkali when using an agar medium. It would seem to be impossible to obtain as good growths on plates over alkali



Figs. 11 and 12.—Growth of the tubercle bacillus on inverted plates, the former over water, the latter over KOH, in a jar through which moist air was passed at a slow speed. The growth is better than that seen in figs. 9 and 10 because of less desiccation.

as on those which are inverted over water. The fact that a good initial growth is obtained on the former, before the effect of desiccation becomes marked, can be considered as proof that CO_2 is not essential to the growth of the tubercle bacillus. Alkali does not inhibit the growth because of removal of CO_2 but rather because of the removal of moisture.

MOISTURE REQUIREMENTS

It is a matter of common observance that a culture of the tubercle bacillus ordinarily does not develop, as a continuous growth, over the entire surface of the medium. No matter how carefully the inoculum is spread over the surface of the agar, the growth is uneven. Some areas may show no development, and this might suggest that many of the transplanted organisms are not viable.

This peculiarity of the organism is largely, if not wholly, dependent on the supply of moisture. As pointed out before, agar is distinctly hydrophilic. It tends to take up moisture from the air, and its surface after a few days becomes relatively dry and hard. Consequently, the isolated organism is placed under a disadvantage which it cannot overcome. On the other hand, the larger masses of the inoculum are able to retain their moisture and can even add to it by drawing on the underlying supply. Hence it is that growth is first recognizable in or about such masses. As a matter of fact, the earliest evidence of growth can be detected on the surface of the water of condensation where the tiniest bit of inoculum can be seen to enlarge before such increase can be recognized on the agar surface.

The tubercle bacillus, like other organisms, is aquatic. It can thrive only when surrounded by moisture and the requisite amount of oxygen. The characteristic wrinkling of a surface growth is indicative of multiplication, not so much on the surface as in the underlying layer where the moisture supply is at its best. The essential reason why the organism does not grow in the deeper layers of broth or agar is the lack of oxygen. When the O_2 concentration in the atmosphere is increased to 80 or 100%, there is a corresponding increase in the amount of O_2 dissolved by the broth or agar. The organism is then able to grow in the depths of either a liquid or a solid medium.

The effect of desiccation can be easily demonstrated by a direct test. Figure 13 serves to illustrate an experiment of this kind in which a current of CO_2 -free air was passed through 4 culture tubes. The air flowed through tubes 1 and 2 at the rate of about 10-15 c.c. per minute

and through tubes 3 and 4 at a slower rate, about 0.1 c.c. per minute. As late as the 9th day, the first pair showed little or no sign of growth, whereas the second pair at that time was very rich. The appearance on the 14th day is that shown in fig. 13.

Three experiments were made to demonstrate that the unevenness of development on the agar surface could be obviated by the introduction of moisture. For each experiment, 12 tubes of glycerol agar were inoculated,

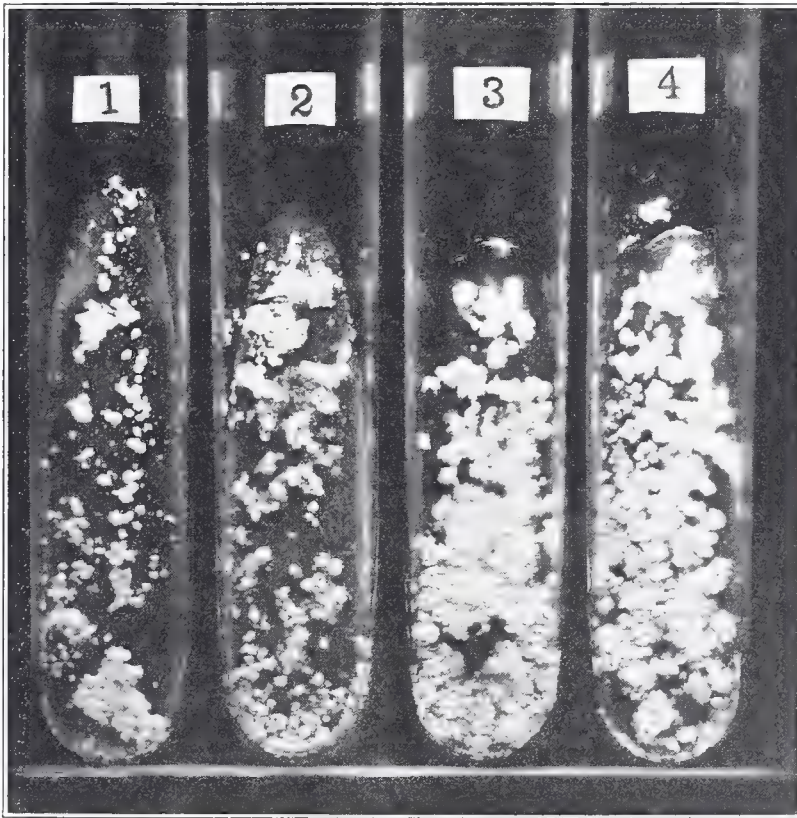


Fig. 13.—Growth of the tubercle bacillus, 14 days at 37 C. Nos. 1 and 2 were rapidly aerated, which resulted in some drying of the surface, and hence in poor growth. Nos. 3 and 4 were aerated very slowly; the surface was very moist and the growth was rich.

closed with sealing-wax and holed. These tubes were provided with side-arms (fig. 2 C, Part I), which were closed with a short piece of rubber tubing and a glass rod. They were kept at 37 C. for 14 days.

A jet of steam, generated in the same way as given under "Aqueous Tension" in Part I, was introduced through the side-arms of Tubes 4-12, so as to cause a good deposit of moisture over the inside of the tubes. Tubes 4 to 6 were given only one steaming. Tubes 7-9 were steamed daily for 14 days. Tubes 10-12 were likewise steamed daily, but, before the treatment, the agar was cooled for a few minutes in ice water. Tubes 1 to 3 were untreated and served as controls.

It was interesting to note that after the first steaming all of the moisture disappeared in a few hours. The moisture was chiefly taken up by the hydrophilic agar and to some extent by the cotton plug. This removal of the moisture occurred regularly until about the 6th steaming. After that some moisture remained on the wall of the tubes for most of the following day. An empty tube which was plugged and sealed in the same manner as above, likewise absorbed all of the moisture deposited by the first steaming. In this case, the cotton plug was the sole absorbent. The moisture from the second and subsequent steamings was not taken up.

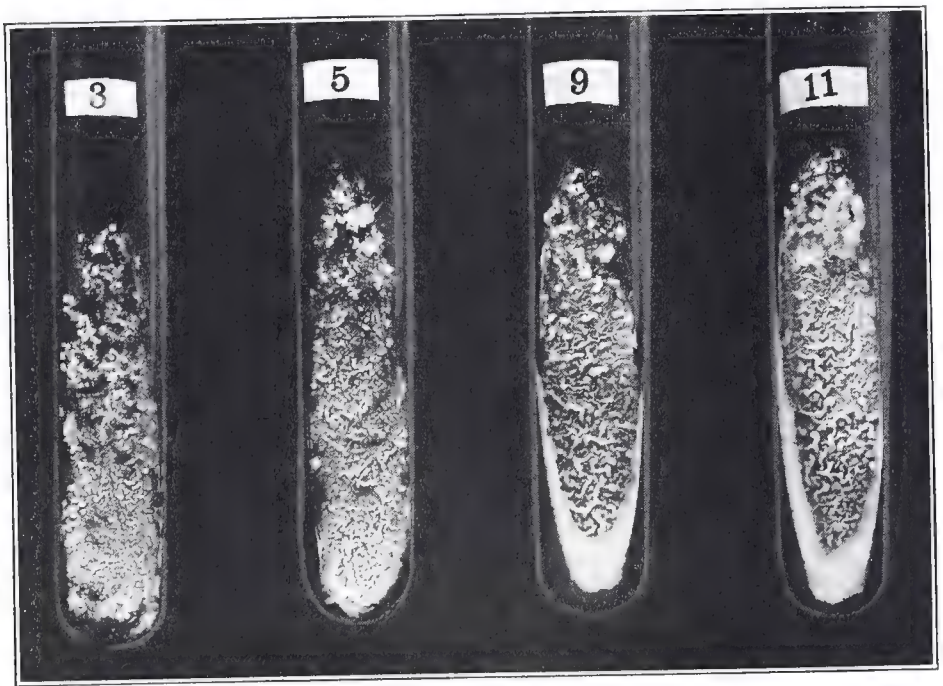


Fig. 14.—Effect of moisture on the growth of the tubercle bacillus, 14 days, at 37 C. Tubes 9 and 11 were steamed daily; note the heavy growth on the surface and its spread over the wall of each tube. Tube 5 was steamed but once, and Tube 3 was not steamed. Note the poor growth and irregular development as compared with that in nos. 9 and 11.

On the 5th day, tubes 1 to 6 showed a dry surface and but a slight growth. The medium in tubes 7 to 12, which were steamed daily, was moist and had a fair growth.

On the 9th day, a fair growth was present in tubes 1 to 6, but it was not as abundant as in the remaining 6 tubes.

On the 14th day, tubes 7-12 were overgrown with a thick, moist, white culture (10 +); tubes 4 to 6, which received but a single steaming,

were less rich (8+); while the unsteamed controls had even less growth (6+). Fig. 14 shows the appearance of the culture in one tube of each set. Unlike the usual culture, which is thin, dry, yellowish and patchy, the steamed cultures were thick, moist and white, and covered the entire surface.

It is evident that, in addition to a good medium, the tubercle bacillus requires an abundance of air and plenty of moisture. These conditions are especially encountered in jar cultures, and there it is possible to observe multiplication as early as the 3rd day. Much the same result can be obtained by "holed" sealing-wax cultures provided the hole is small. It has been shown that one can obtain rich cultures in from 7 to 10 days when the requirements of air and moisture are satisfied.

MERCURY TENSION

In the earlier part of this paper it was pointed out that ordinary culture tubes, closed with sealing-wax, usually developed a good growth, whereas when such tubes were attached to manometers, no growth resulted. It seemed that if the sealing-wax permitted air to pass through it in the unattached tubes, it should do likewise with those connected with manometers. Seeking for the cause of this inhibition, attention was directed to the Hg column in the manometer. Possibly, it disengaged sufficient Hg vapor to inhibit the growth. To test this point, a cylinder of gold foil, about 5 cm. long, was inserted into the tip of the manometer so as to absorb any Hg vapor that might come over. When tubes were attached to such manometers, they likewise failed to develop any growth, thus demonstrating that the failure was not due to that factor.

The question of Hg tension, however, was subjected to another test. Each of 4 side-arm tubes (fig. 2 C, Part I) were inoculated and closed with sealing-wax. Into a short tube, bent at right angles, and closed at one end, some Hg was introduced, and this tube was attached to the side-arm by means of a No. 25 rubber stopper treated with glycerol. As a control, 4 other tubes were similarly equipped, except that the bent tube contained water instead of Hg. The 8 tubes when incubated for 21 days gave exactly the same result—no growth. This again seemed to demonstrate that the failure to obtain growth was not due to the vapor tension of Hg. At this point of the work, it was recognized that the real cause of the failure was due to lack of air. To prove that such was the case in this experiment, 2 of the tubes with Hg and 2 of the tubes with water in the side-arm were "holed," whereas the other 4 tubes were not. Within a week, a good growth developed in the "holed" tubes, regardless of whether Hg was present or not. The tubes that were not "holed" failed to show any growth even for as long as 64 days.

The foregoing experiment therefore confirmed the need of an abundant air supply. It seemed to demonstrate that Hg vapor was a negligible factor as regards the inhibition of the growth of the tubercle bacillus. This was certainly true as regards the action of the Hg in the manometric tube or in the side-arm, but it was not true for other conditions, as was learned later and after many annoying failures.

In the course of the study of the organism in varying tensions of O_2 , or of CO_2 , it not frequently happened that the cultures in 1 or 2 jars failed to grow, whereas in other jars, prepared at the same time and inoculated with the same material, the growth was rich. Here it was not a question of the lack of oxygen.

Again, it was noted that a white culture developed in 90% O_2 when transplanted and returned to the same tension of O_2 grew as a grayish-black, moist mass. On 3 separate occasions, this black growth was obtained. It seemed as if oxidation products resulted from prolonged contact with an atmosphere having a high O_2 content.

Even more striking was the behavior of the transplants made from such a black culture. Thus, in one experiment, 6 transplants were made. Two of the tubes were closed with sealing-wax and "holed"; these produced rich, pure white cultures. Examined from the under side, it could be seen that the original black inoculum persisted. Two of the tubes which were placed in a jar in an atmosphere of 98% O_2 , developed about 20 large white colonies, but the center of each showed the original black spot. The 3rd pair of tubes which were placed in a jar, with an atmosphere containing 90% O_2 and 10% of CO_2 , gave good but dirty, grayish-black cultures. The reversion to the white type, in the case of 4 of the cultures, called for an explanation.

It was noted that the earliest indication of an oncoming black strain was the presence of a pearly, semimetallic luster rather suggestive of a thin film of HgS . In time, the conviction grew that the color of the black culture was actually due to HgS , and, if so, it should be capable of demonstration.

This view was supported by the fact that a few globules of Hg could always be found on the bottom of the jars in which the tubes showed either no growth or one that was black. The presence of the Hg in these jars was due to accidental introduction by the method then used for sampling the gas content.

As a preliminary test to determine whether or not Hg was responsible for the inhibition and blackening of a culture, 2 bulb tubes of 100 c.c. capacity

(fig. 2 A, Part I) were inoculated with the normal stock culture. A drop of Hg was placed in each bulb, after which the tubes were closed with sealing-wax and "holed." As controls, 2 ordinary tubes were inoculated and sealed in the same way. In 14 days, the control tubes showed the usual rich growth (10+), whereas the bulb tubes, with Hg, had at most 20 or 30 minute pinheads which showed a thin but distinct film of HgS. At the end of 28 days, the latter tubes gave no evidence of growth, but the medium had taken on a decided dark, smoky color. On the 35th day, the Hg was poured out of the tubes. Within a week, white edges appeared around the black pinheads, and this white growth was considerably increased during the following month. This experiment, therefore, demonstrated that the Hg vapor did inhibit growth, and that it was the cause of the blackening of the culture and of the medium. The drop of Hg, as stated, was in the bulb and at no time in contact with the medium.



Fig. 15.—Effect of Hg vapor on the growth of the tubercle bacillus, 28 days at 37 C. No. 1 was in a jar with Hg; result complete inhibition. Its control, no. 3, was likewise in a jar but without Hg—rich growth. Nos. 5 and 7 were bulb tubes closed with sealing-wax and holed. The former had a drop of Hg in the bulb, while the latter had none.

In the next experiment, 4 bulb tubes and 6 ordinary tubes were inoculated from a stock culture. Into the bulb of 2 of the former (nos. 5 and 6) a drop of Hg was introduced. The other 2 bulb tubes (nos. 7 and 8) received no Hg. These 4 bulb tubes and 2 of the straight tubes were then closed with sealing-wax and holed. Two other straight tubes (nos. 1 and 2) were set in a Novy jar on the bottom of which had been placed about 1 c.c. each of water and of Hg. Tubes 3 and 4 were placed in a similar jar, which, however, had no Hg. The jars were filled with pure outside air, then closed and set aside at 37 C.

The results of this experiment were most satisfactory. The tubes in the Hg jar, as well as the bulb tubes with Hg, showed no growth in 28 days, but the medium took on the characteristic grayish, smoky appearance. By contrast, the 6 control tubes, which were not exposed to the vapor of Hg, gave rich white cultures (10+) by the 14th day. Fig. 15 shows the appearance of 2 of the tubes (nos. 1 and 5) which were exposed to the Hg vapor and of 2 corresponding controls (nos. 3 and 7).

These experiments proved conclusively that the two anomalies mentioned above were wholly due to the action of Hg vapor. When the inoculated mass is small, the Hg vapor inhibits the growth completely. Larger masses, especially when under the stimulus of high O_2 tension, are able to increase and give rise to an appreciable growth which, however, takes on a dirty black color due to the formation of HgS. In either case, the medium assumes a decided smoky appearance.

It has been shown that the effect of Hg vapor is not in evidence when the Hg is contained in the manometric arm, or in the side-arm of the culture tube. This is probably due to a lack of diffusion of the vapor from such narrow tubes. When, however, the Hg is in a bulb or in a jar, the maximal saturation of the whole air volume readily results, and consequently the full effect of that concentration is seen in the exposed culture. The tubercle bacillus because of its slowness in growth shows the maximal effect. No inhibition is observable with rapidly growing organisms such as *B. coli*. But prolonged exposure of such cultures to the Hg vapor results in blackening of the growth and darkening of the medium.

The presence of Hg in the black culture of the tubercle bacillus was clearly proved. For this purpose, the culture was taken up with water and treated with nitrohydrochloric acid on the water-bath. The clear liquid, obtained after repeated evaporation and filtration, was concentrated to a small volume. The tip of a finely pointed copper wire, when suspended in this liquid, became covered with a minute, grayish-white deposit of Hg.

The presence of sulphur in the tubercle bacillus has been something of a mooted question. In the light of these results, it seems that there can no longer be any doubt on this point.

Existing figures for the tension of Hg vapor vary considerably, as may be seen from the following values, which are those given for the tension at 40 C.:

Regnault	0.0767 mm.
Hagen	0.033 mm.
Hertz	0.0063 mm.
Ramsay and Young.....	0.0008 mm.

Consequently, the calculated amounts of Hg (mg.) present in a cubic meter of air are likewise different. Two attempts have been made to determine directly the amount of Hg present. Renk¹⁰ found 10.4 mg. at 20 C., and 16.8 mg. at 30 C.; Kunkel,¹¹ by a different method, obtained irregular values, but the average of 4 determinations at 20-23 C. was 9.4 mg. It does not appear that any determination has been made for 37 C., but on extending the curve given by Renk a probable value of 22 mg. may be arrived at. Assuming that 25 mg. of Hg are present in a cubic meter of air at 37 C., the concentration is that of 1 mg. of Hg in 40 liters of air.

VIABILITY AND VIRULENCE

The question of the viability of the tubercle bacillus must be considered in connection with the composition of the gaseous content of the culture tube. The assertion that 10% of CO₂ is tuberculocidal is certainly not true in the presence of oxygen. Given the necessary O₂ supply, the organism will multiply, as has been shown, in concentrations up to 90% of CO₂. Just what would be the effect of different percentages of CO₂, in the absence of O₂ remains to be determined. There is some reason to believe that, in cultures, it is the absence of O₂ which is the chief factor in destroying the organism.

It has been shown that a freshly inoculated tube, when sealed and attached to a manometer, produced no visible growth, although a good negative pressure developed. At the end of a month, at 37 C., such tubes contained about 9 to 12% of CO₂ but no O₂. At this time, the original inoculum had not visibly increased. To test its viability, the tubes were disconnected from the manometer and set aside at 37 C. Such tubes often failed to develop growth, thus showing that the inoculated material had died. Death of the culture, however, could not be established with certainty unless free access of air was provided either through an open side-arm or through an opening in the sealing-wax.

¹⁰ Arb. a. d. kaiserl. Gsndhtsamte. 1889, 5, pp. 113-138.

¹¹ Kunkel, A. J., and Fessel, F.: Verhandl. der Physik.-Med. Gesellsch. zu Würzburg, N. F. 1899, 33, (1)—(13).

Of special interest here is an experiment in which 20 tubes were inoculated, closed with sealing-wax and kept at 37 C. The closure was so well done that at the end of 21 days there was no sign of growth in any of the tubes. The odd numbered tubes were then holed; within a week thereafter they all showed good cultures, and in 2 weeks they became rich (10 +), while the tubes which had not been holed remained practically negative. The latter tubes were in turn holed on the 35th day after inoculation, and failed to grow. In this experiment, then, the inocula in 10 tubes died out in from 21 to 35 days.

Experiments such as the above should be repeated with pure nitrogen, or with air, in the presence of alkali, in order to ascertain just how much of this effect is due to CO_2 .

Another phase of the question of viability concerns that of a fully developed, holed culture which is kept at 37 C. To test this point, cultures were selected which had been in the incubator for 8, 12, 16 and 20 weeks, respectively. These were holed at the time of inoculation and were very rich. From each of these tubes, 2 subcultures were made, sealed and holed. The transplant from the 8 weeks' culture developed the usual rich growth, whereas those from the 12, 16 and 20 week tubes failed completely, though under observation for nearly 4 months. The material in these tubes was very granular, and some slight desiccation was in evidence.

The effect of desiccation could be excluded by using tubes which were developed and kept in anaerobe jars. While no special tests were made to determine this point, mention may be made of the fact that cultures which were developed and kept in jars with 100% O_2 , or 90% CO_2 , gave rich subcultures when transplanted on the 59th day.

It may also be added that the black culture, due to exposure to Hg vapor, may remain viable for a considerable period of time. Thus, such a culture developed in 100% O_2 and transplanted on the 56th day gave rich growths. One of these subcultures, however, made into 90% O_2 and 10% CO_2 , was found to be dead on the 57th day. Whether the loss of vitality in this case was due to the presence of the CO_2 or to the prolonged contact with the Hg vapor in the jar was not determined.

Virulence.—The stock culture of the tubercle bacillus used in this study was highly virulent. When injected intraperitoneally into guinea-pigs it usually was fatal in about 3 weeks. Only a limited number of tests for virulence were made with cultures developed under different

gas conditions. This phase of the work, however, is deserving of careful study.

The black culture mentioned above as having been developed in a jar with 100% O_2 , on the 56th day was injected into 2 guinea-pigs. These died on the 26th and 33rd day, with extensive tuberculous involvement, enormously enlarged mesenteric glands, miliary tuberculosis and peritonitis. The culture used was a second generation, 56 days old, in 100% O_2 . Its black color was due to the Hg present in the jar. The first generation was developed for 21 days only and was pure white. Evidently the presence of HgS in this culture did not interfere with its viability or its virulence.

By way of contrast it may be added that the injection of a pure white culture developed in 100% O_2 for 58 days produced death in 2 guinea-pigs in 67 and 69 days. The mesenteric glands were hardly affected, but the spleens were greatly enlarged and highly tuberculous; the lungs and the liver were likewise heavily involved. A striking feature in both animals was the development of lesions involving the eyelids, nose and genital labia. These were suggestive of skin tubercles, but a pathologic examination made by Dr. C. V. Weller gave no evidence of this condition. They were essentially horny warts and probably in nowise connected with the injection of this culture.

Of further interest is an experiment in which 2 guinea-pigs received subcutaneous injections with a pure white culture grown in a jar which contained, at the start, 85.8% CO_2 and 11.4% O_2 . At the end of 59 days, analysis showed 96.05% of CO_2 and 1.5% of O_2 . The growths were then used for subcultures and for inoculation of the guinea-pigs. It may be added that the subcultures became rich in 2-3 weeks. One of the guinea-pigs died, following abortion, on the 57th day after inoculation. The spleen and lungs were highly tuberculous, while the liver showed extreme fatty degeneration. The other guinea-pig died on the 179th day of pneumonia. Apart from the caseous mass under the skin, there was practically no gross evidence of tuberculous involvement.

This experiment shows that the culture developed in an atmosphere of 85 to 96% CO_2 over a period of 59 days was viable and was virulent for one animal.

SUMMARY

This paper concerns the study of a single strain of human *B. tuberculosis* grown on certain solid mediums.

By the use of exact methods, the gas changes of the organism have been followed under varied conditions.

Its average corrected real respiratory quotient, when grown on glycerol agar, was 0.836; on glucose agar, 0.992; while on rabbit serum agar, it was 0.904. The quotient was not influenced by the growth of the organism in high or low O_2 , or in high CO_2 tensions. The theoretical value is, for glycerol, 0.857; for glucose, 1.

The analyses demonstrated that the gas exchange was greatest when the organism was grown on glycerol agar (table 5). A good growth was obtainable on glucose agar. Also, that in order to obtain a rich growth in a single tube, about 100-150 c c. of oxygen must be provided. This means a supply of ordinary air corresponding roughly to 500-700 c c. at 37 C. and 750 mm. The analyses also demonstrated that the yield of CO_2 was slightly less than the amount of O_2 consumed. In tests with air, in jars, as much as 150 c c. of unreduced CO_2 were produced by a single culture.

In a closed, ordinary culture tube the oxygen was removed in a few days by the inoculum, and no visible growth resulted. A very slow growth, or none, in tubes closed either in the flame, or with sealing-wax, paraffin, or rubber stoppers means an insufficient supply of O_2 .

When all of the O_2 (20.9%) was consumed by the culture, the total CO_2 yield reached about 17.5%. This does not mean O_2 retention by the cell. It merely expresses the fact that in the combustion of glycerol the volume of CO_2 produced is less than the volume of O_2 consumed, as expressed by the ratio $\frac{6}{7}$ and, hence, by the quotient 0.857. One-seventh of the O_2 consumed combines with H_2 to form water and, as a result, a corresponding negative pressure develops.

When using glycerol agar as the culture medium, the manometer developed a negative pressure which reached a constant level as soon as the O_2 was entirely consumed. Some of this negative pressure was due to loss of CO_2 by solution in the medium. There was an additional loss of CO_2 when rubber stoppers were used.

Rich cultures developed in tubes which were attached to manometers, provided that they were evacuated and refilled with pure air as often and soon as O_2 removal was indicated. After 5-10 refills, the O_2 thus supplied was enough to provide a good growth. The volume of growth was strictly proportional to the amount of O_2 consumed, provided the O_2 tension was constant.

When such tubes were refilled so as to contain increasing tensions of O_2 , the manometers responded with increased negative pressures, and

the analyses showed corresponding high yields of CO_2 . Thus, a tube charged with slightly less than 100% O_2 showed a pressure of — 162 mm. and yielded 86% of CO_2 .

The optimal concentration of O_2 was at about 40-50%. Above that limit, growth was less abundant. In 100% oxygen, the growth developed as isolated, thick, moist, white colonies; at the end of 58 days, such a culture was infective.

Growth occurred when the O_2 tension was decreased below that in air. In atmospheres containing 10, 6, 3, 1 and 0.5% O_2 , the rate of growth was correspondingly retarded. An essential condition for good growth under diminished tension is that the vessel shall contain at least 100 c.c. of O_2 . Growth continues "until the last atom" of oxygen is consumed.

With a like consumption of O_2 (100 c.c.), the growth mass is proportional to the O_2 tension originally present in the container. The higher the tension (up to certain limits), the greater is the growth mass. The lower the tension, the smaller and poorer is the growth. (Figure 7).

In atmospheres containing 10 to 50% of CO_2 , the growth of the tubercle bacillus was not inhibited. In concentrations of 60% or more there was some inhibition, but fair growth was obtained in 90% CO_2 . The growth which developed in 85-96% CO_2 for 59 days was viable and infective.

The tubercle bacillus as it grows produces CO_2 , but the removal of this respiratory CO_2 as fast as it is produced does not stop growth. Whatever growth inhibition does occur when a culture is kept over alkali is the result of desiccation of the surface of the medium.

Moisture sufficient to wet the surface of the medium was found to be an important factor in securing rich and even growths.

The vapor tension of Hg was sufficient to inhibit the growth under ordinary O_2 tension. Under high O_2 tension the organism multiplied in the presence of Hg vapor, but the resultant growth was grayish-black and infective.

The best results as regards rapidity of growth and richness of culture were obtained with a meat extract medium containing 1% agar and 5% glycerol. Moisture can be supplied by a fine jet of steam introduced into the cotton plug. After inoculation such tubes were closed with sealing-wax and holed by means of a hot platinum wire. This method of cultivation and sealing is recommended, since in from 7 to 10 days it gave good growths.

The slow multiplication of the tubercle bacillus in the body is explainable from the standpoint of growth in diminished O_2 tension. An indefinite supply of O_2 under a tension corresponding to a few mm. of Hg will probably enable the organism to grow, though very slowly. The "rest cure" and rich diet in checking the progress of the disease probably act by reducing to a minimum the available O_2 supply in the tissues.

CLASMATOCYTES AND PASSIVE IMMUNITY TO STREPTOCOCCUS INFECTION

VI. STUDIES IN STREPTOCOCCUS INFECTION AND IMMUNITY*

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In our latest study¹ of experimental streptococcus infection in the rabbit, we have pointed out relations that exist between conditions of natural resistance and the absolute number of clasmatocytes (tissue macrophages) that are present at the site of inoculation. The exudate provoked in the pleural cavity of the rabbit varies in the relative and total number of its constituent cells in accordance with the substance employed. Thus it was found that infusion broth and dilute (1%) egg white would in 24 hours lead to a large increase in the clasmatocytes present, whereas aleuronat and concentrated egg white lead to relatively small numbers of clasmatocytes and a large increase in polymorphonuclear cells. Whenever the total number of clasmatocytes in a pleural cavity was raised from its normal of 300,000 to something like 4,000,000 by injections of this sort, the animal was found to be protected against at least 100 M L D of our streptococcus "H." There was one exception, when diatomaceous earth was used and a large number of clasmatocytes resulted but no protection was afforded. In this instance, the cells were abnormal in appearance and staining reactions. On the other hand, the number of polymorphonuclear cells seemed to bear no definite relation to protection.

Further study showed that the injection of streptococcus into a broth prepared (protected) animal produced a characteristic and rapid clasmatocyte crisis coincident with sterilization of the cavity, whereas in the normal unprepared animal the clasmatocytes dropped below normal and then rose slowly to a maximum in from 24 to 48 hours, which was distinctly below that reached by the prepared animal in three hours. In the normal animal, the bacteria increased progressively hour by hour

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* Assisted by Michael J. Bent, Fellow of the National Research Council.

† Preceding studies in this series are: Gay, F. P., and Stone, R. L.: *Jour. Infect. Dis.*, 1920, 26, p. 265. Gay, F. P., and Morrison, L. F.: *Ibid.*, 1921, 28, p. 1. Gay, F. P., and Rhodes, B.: *Ibid.*, 29, p. 217; *ibid.*, 1922, 31, p. 101. Gay, F. P.: *Jour. Immunol.*, 1923, 8, p. 1. Gay, F. P., and Morrison, L. F.: *Jour. Infect. Dis.*, 1923, 33, p. 338.

¹ Gay and Morrison: *Jour. Infect. Dis.*, 1923, 33, p. 338.

until death. The time relations in the two series of animals in respect to polymorphonuclear output was similar, although the same maxima were reached in each. For reasons already given, the clasmatoocytes were regarded as more important than the polymorphonuclear cells in this form of protection.

A preliminary survey of the relation of clasmatoocytes to passive immunity was also made. We had learned from our previous work² that a strictly homologous antiserum from the rabbit will, when injected into the pleural cavity simultaneously with the streptococcus, frequently prevent the evolution of empyema. Normal rabbit serum had no such effect. We later found that the injection of normal rabbit serum alone into the pleural cavity produces a clasmatoocyte increase as great as that produced by rabbit antistreptococcus serum. Suggestive evidence was offered, however, that the outcome of infection of these cavities is quite different in accordance with whether normal or immune serum is employed.

It is our purpose in this communication to discuss more fully the mechanism of passive immunity against experimental streptococcus empyema in the rabbit.

METHODS AND TECHNIC

The methods we have employed have been detailed in previous articles, particularly in the latest one by Gay and Morrison.³ In general, the methods there described are continued unless otherwise stated.

1. The method described of estimating viable streptococci in a culture by plating continues to give consistent results.

2. The minimal lethal dose intrapleurally as determined for 1922-1923 in Washington rabbits was 0.00001 c.c. of a 24-hour broth culture, or about 1,300 organisms. We have continued to utilize successive pleural fluids from rabbits that have died of empyema with passage streptococcus pyogenes "H," employing during the past year "pleural generations" from No. 64 to No. 72, as a source of inoculation material for further cultures designed to produce pleurisy. During the past year in New York City, fewer bacteria have sufficed to kill an adult rabbit. Either the virulence of our organisms has increased or the susceptibility of local rabbits is greater. Thus, in two separate series, in January and March, 1924, the minimal lethal dose intrapleurally had fallen to about 15 streptococci (dilution of 24-hour broth culture, 10^7) and correspondingly⁴ the intravenous lethal dose had dropped from 0.1 c.c. to 0.01 c.c.

3. Staining and classification of clasmatoocytes. We have continued to use neutral red as a differential supravital stain for cells which we classify as clasmatoocytes. Our method in employing this stain has changed somewhat. We now prefer the National Aniline and Chemical Company stain which is

² Gay and Stone: *Ibid.*, 1920, 26, p. 265.

³ *Ibid.*, 1923, 33, p. 338.

⁴ Gay and Rhodes: *Ibid.*, 1922, 31, p. 101.

more active than the Grüber stain previously employed. The stain is prepared in a 1% solution in sterile distilled water and diluted further to 1:2,000 to 1:4,000 in sterile saline just before use. The final dilution of dye with equal parts of exudate ranges, therefore, from 1:4,000 to 1:8,000. Only those cells that take up this dye in vacuoles with sharply delimited margins are classified as clasmatoocytes. Check stains were made on fixed preparations by hematoxylin and eosin (as previously described) by Wright or by the Proca-Kayser method.⁵ These control stains usually show more large mononuclear cells than the neutral red and also, under certain conditions, small mononuclear cells which have been tentatively classified as lymphocytes. These cells may well belong to the same series as typical clasmatoocytes. In other words, the evidence which we have assembled in pointing out the significance of clasmatoocytes must distinctly underestimate their actual numbers in any given experiment.

4. Antistreptococcus serums employed. These serums were obtained from our Washington series of rabbits that had been immunized by intradermal, intrapleural, or intravenous routes, and subsequently tested with multiples of the lethal dose for comparative purposes by the same or a different route. The survivors that gave evidence of a complete active immunity under the conditions employed were bled 10 days to 2 weeks later, and their serums inactivated at 56 C. and stored for from 6 to 8 months. The separate serums were tested for their ability, when injected in doses of 1 to 3 c.c., 24 hours before and again simultaneously with 200 or more pleural lethal doses of *Streptococcus* "H," to sterilize the pleural cavity within 24 hours. Two or more such serums were pooled, and pooled lots of this sort were used, each in a given experimental series. Normal rabbit serum, heated to 56 C., was employed as control.

THE DESTRUCTION OF STREPTOCOCCUS IN NORMAL SERUM AND IMMUNE SERUM PREPARED PLEURAL CAVITIES

We have already referred to the cell content of pleural exudates produced in rabbits by the injection of 3 c.c. of inactivated normal rabbit serum, or of the same amount of rabbit antistreptococcus serum. These exudates were found not to vary to any extent in total numbers of clasmatoocytes or of polymorphonuclear cells, as is shown in table 1.

TABLE 1
AVERAGE CELL CONTENT OF RABBITS' PLEURA 24 HOURS AFTER INJECTION OF 3 C.C. NORMAL
OR IMMUNE RABBIT SERUM

No. of Animals	Serum Injected	Total Cells	Total Clasmatoocytes	Others
4	N. S. 3 c.c.	4,500,000	2,446,450	2,053,550
5	I. S. 3 c.c.	3,254,000	2,100,530	1,153,470

For our present purpose, we have finally reduced the amount of serum injected to prepare the cavity, employing 1 c.c. only. Moreover, we have estimated the cells in the cavity 18 hours later instead of 24

⁵ Kayser: *Centralbl. f. Bacteriol., I. O.*, 1912, 62, p. 174. Stain: Methylene blue, from 2 to 3 minutes. Wash. Stain with dilute carbol fuchsin (1:10) from 5 to 10 seconds. Wash.

hours later. The results assembled in table 2 show again that the normal and immune serums give similar results in respect to the different cells. Lymphocyte counts (from fixed preparations) are also given here.

On comparing the figures in table 2 with those in table 1, it appears that the total cell count is larger and that this increase is largely due to a greater number of polymorphonuclears, as we should expect in an earlier exudate. Precisely how the lymphocytes function is not clear, but at least in the previous counts (table 1) they were included in the count of the polymorphonuclear cells.

We have found that the best method to demonstrate the protective action of immune serum is to inject 1 c.c. 18 hours before a second injection of 1 c.c. which accompanies the infecting dose of streptococci (150 to 800 M L D⁶) which are simply suspended in the immune serum or normal control serum in a volume so small as not to disturb the total

TABLE 2
AVERAGE CELL CONTENT OF RABBITS' PLEURA 18 HOURS AFTER INJECTION OF 1 C.C. NORMAL OR IMMUNE RABBIT SERUM

No. of Animals	Serum	Total Cells	Clasmato-cytes	Polymorpho-nuclears	Lympho-cytes
3	N. S.	7,478,666	1,092,746	4,063,320	2,322,600
3	I. S.	5,888,333	1,015,464	2,982,024	1,890,845

quantity relations. We may anticipate by saying that this previous and simultaneous injection of rabbit antistreptococcus serum suffices to sterilize the cavity of the rabbit in from 1 to 3 hours. In rabbits that have received previous and simultaneous injections of normal rabbit serum, the bacteria increase rapidly for about 24 hours and then usually diminish to sterilization in 2 or 3 days. In the original study of Gay and Stone, immune rabbit serum (1 c.c.) would sometimes cure streptococcus empyema when given with the infecting culture and followed by another dose. The same doses of normal serum did not cure. In the later work of Gay and Morrison, it was noted that the few control animals of that series treated with normal serum, which were allowed to progress after infection without examination, did not die, and the observation of an abscess in the pectoral muscles with a clean pleura when they were killed a number of days later, led us to assume that the injection had been faulty, although such failure rarely occurs in experienced hands. We now know that, without doubt, the normal serum had

⁶ Actually 2,000 to 13,000 organisms, or 0.00002 c.c. of a 24-hour broth culture.

led to a cure, although a delayed one. As a control to our present series, we prepared 7 rabbits with 1 c c. of normal serum, and on the following day gave the same amount with the addition of 200 lethal doses of streptococcus. Of these 7 animals, 5 recovered and only 2 died of pleurisy.

As indicated above, there is a critical sterilization of the pleural cavity in the animal that has received previous and simultaneous injections of immune serum. This fails to occur in the normal serum control. We have just seen that the cell count in normal animals and in those treated with immune serum is essentially the same at the time when the second dose of serum including the streptococcus is given. It was essential to know whether the second serum dose in itself, without bacteria, leads to different cell counts in accordance with whether it is normal or immune.

Table 3 shows the results in this respect in a limited series of animals given a 1 c c. dose of normal or immune serum and into which the same

TABLE 3
CELL CONTENT IN ANIMALS RECEIVING A 1 C C. DOSE OF NORMAL OR IMMUNE SERUM, THE
SAME SERUM BEING REINJECTED 18 HOURS LATER

No. of Animals	Serum	Total Cells	Clasmato- cytes	Polymorpho- nuclears	Lympho- cytes
4	N. S.	13,626,000	1,266,740	9,981,620	2,377,640
3	I. S.	29,288,666	1,571,813	25,868,173	1,848,680

serum is reinjected 18 hours later, examination being made between 1 and 2 hours later.

The figures in table 3 show that a second injection of serum does increase the total number of cells in both normal and immune series beyond that produced by a single injection. No marked change has taken place in the clasmatocytes, but the polymorphonuclears are doubled following the second dose of normal serum, and increased 8 times in the immune serum series.

We may now proceed to discuss the results observed when the normal or immune serum prepared rabbit pleura is reinjected with the same serum containing 200 or more lethal doses of streptococcus. Several lots of pooled immune serum have been tried, and a more or less extensive series of time intervals chosen with each, in order to compare the results in cell counts and corresponding decrease or increase in the number of bacteria. It will be appreciated that even with 3 workers, the total number of animals that can be examined, following infection at the same

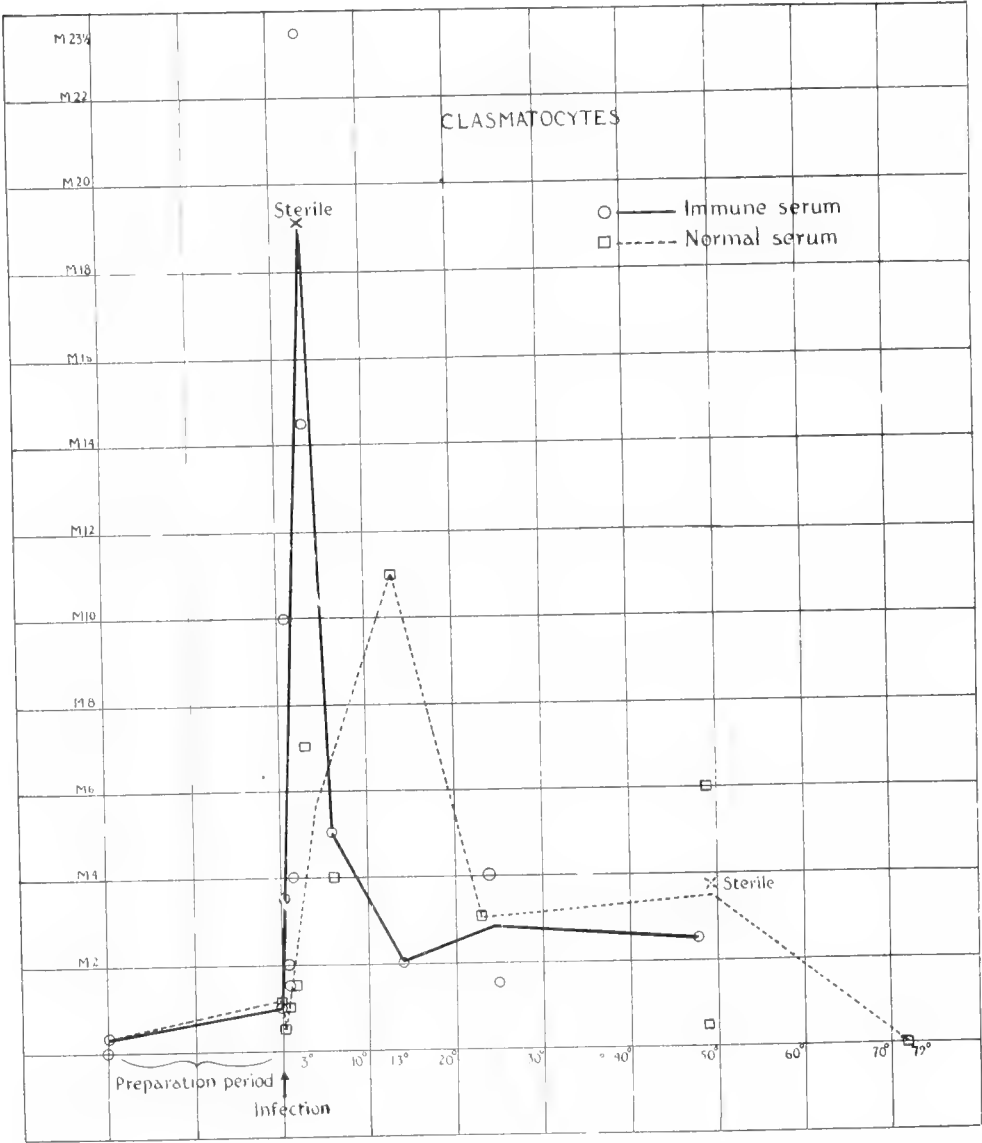


Chart 1.—Clasmatocytes in pleural cavity following preparation and simultaneous injection of normal or immune serum with streptococcus.

time, is limited, but with the precision of our broth cultures, relative constancy of the number of lethal doses was obtained, and with a given immune serum experiments could be made to overlap on successive days, although fresh cultures were, of necessity, employed and varied somewhat in the actual number of bacteria.

A shorter experimental series is given in table 4, which represents the effect produced with from 5,400 to 9,400 streptococci (360-625 M L D) injected into the pleural cavity of rabbits with 1 c c. normal or immune rabbit serum, and preceded 18 hours by injections of the same serum.

It is evident that rapid sterilization of the cavity when immune serum is used is coincident with a crisis in both clasmatoocytes and polymorphonuclears and lymphocytes. In the normal serum animals, there is a

TABLE 4
CELL CONTENT IN RABBITS RECEIVING INJECTIONS OF STREPTOCOCCI AND NORMAL OR IMMUNE RABBIT SERUM

Rabbit	Killed	Bacteria	Total Cells	Clasmato- cytes	Polymorpho- nuclears	Lympho- cytes
Normal Serum Preparation:						
577	40 min.	$\times 0.3^*$	3,680,000	147,200	3,312,000	220,800
575	1½ hrs.	$\times 1.1$	9,240,000	277,200	8,408,400	554,400
571	3½ hrs.	$\times 3$	85,400,000	854,000	83,692,000	854,000
568	12½ hrs.	< 117.947	129,206,000	2,584,120	124,037,760	2,584,120
Immune Serum Preparation:						
578	40 min.	0.004	30,258,000	4,528,700	22,088,340	3,631,560
576	1½ hrs.	0	41,800,000	4,180,000	31,804,000	5,816,000
572	4 hrs.	0	7,520,000	1,203,200	5,865,600	451,200
547	13 hrs.	0	3,200,000	512,000	1,870,000	818,000

* Indicates the multiple of the original number of bacteria injected.

sharp fall in the clasmatoocytes and lymphocytes from the number normally present 18 hours after a first injection of serum, followed by a slow rise in these cells at a later period (from 12 hours on). The polymorphonuclears do not fall in the normal serum animal, nor do they rise as sharply as in the immune serum animal. The streptococci are killed rapidly in the immune serum animal, so that cultures are sterile in 1½ hours. In the normal serum animals, they increase rapidly up to 12 hours. As we have stated, such animals usually recover and their cavities become sterile in from 2 to 3 days.

The more complete picture of the process is given in graphic charts of another and more extensive series of animals (charts 1 and 2).

In this experiment, as in the other, a single pooled immune serum was employed. The animals, whether normal or immune serum pre-

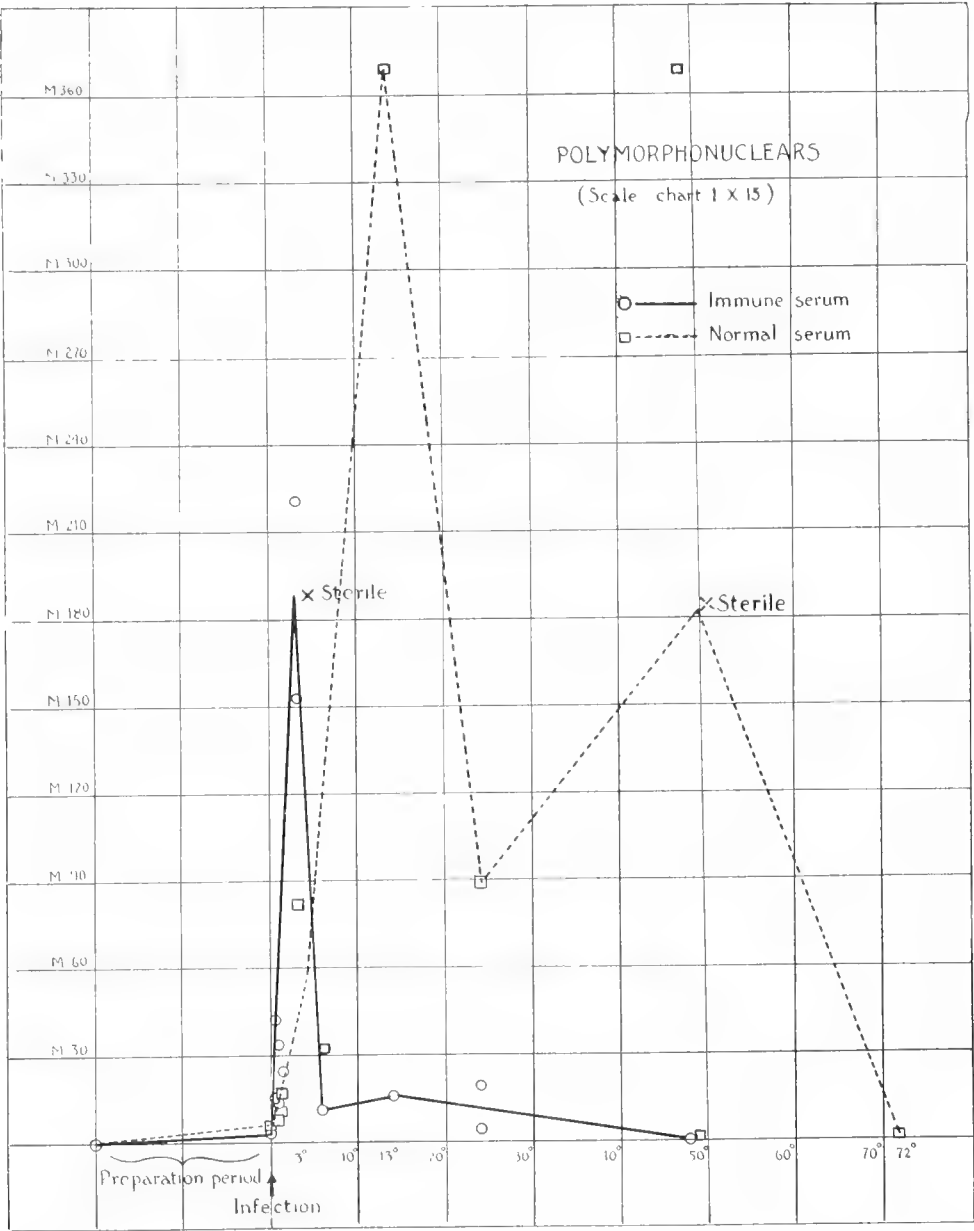


Chart 2.—Polymorphonuclears in pleural cavity following preparation and simultaneous injection of normal or immune serum with streptococcus.

pared, were infected in convenient groups, with multiples of the lethal dose, which varies from 136 to 866.

Each animal is recorded on the chart, and the line indicates the average when two or more animals were killed at the same time interval. The cell response in normal serum animals is compared with the immune serum prepared animals in respect to clasmatocytes (chart 1) and polymorphonuclears (chart 2). The point of complete sterilization of the pleural cavity in both normal and immune series is indicated in both charts.

The clasmatocytes reach their maximum in the immune series in three hours, and this is coincident with sterilization of the cavity. Clasmatocytes in normal serum animals show an initial drop and reach a maximum, which is only one-half that of the immune serum animals at a much later period (13 hours). This is not coincident with sterilization, which occurs 36 hours later.

The polymorphonuclear cells reach their respective maxima at the same periods as do the clasmatocytes in both series, but are over twice as high in the normal, in which only a belated sterilization occurs, as in the immune.

MECHANISM OF CRITICAL STERILIZATION OF THE PLEURAL CAVITY WITH IMMUNE SERUM

As already stated, we have not succeeded in sterilizing the pleural cavity in the manner outlined unless immune serum in the dosage employed is reinjected with the infecting dose of streptococcus as well as preceding it. A single preceding dose of immune serum does not accomplish the desired result. The preceding dose of either immune or normal serum in 18 hours causes an equal rise of both clasmatocytes and polymorphonuclears. In such a "prepared" cavity not only are streptococci accompanied by immune serum destroyed, but streptococci that have been sensitized or tropinized by the immune serum are likewise killed. Unless the cavity is prepared by either normal or immune serum, sensitized bacteria are not killed. These results are outlined in exper. 1.

Exper. 1.—Preparation: Rabbit 379 was given 1 c.c. normal serum intrapleurally, 20 hours before infection. Rabbit 380 was given 1 c.c. immune serum intrapleurally, 20 hours before infection. Rabbit 370 was given 1 c.c. immune serum intrapleurally, 23 hours before infection. Rabbit 381 was given 1 c.c. immune serum intrapleurally, 20 hours before infection. Rabbit 657 was given no preparation.

Four of these animals were each given intrapleurally, 20 to 23 hours later, 200+ M L D of passage streptococcus "H" in a volume of 1 c.c. of broth after contact with an excess of immune serum A, for 30 minutes at room temperature and subsequent washing. The animals were killed 3 hours later, and cultures from their respective cavities showed total numbers of bacteria as follows: rabbit 379 (normal serum prepared), sterile; rabbit 380 (immune serum prepared), streptococci reduced to one twenty-fifth of original number; rabbit 370 (immune serum prepared), sterile; rabbit 657 (no preparation), streptococci increased 596 times. Rabbit 381 was given 200+ M L D of the same culture of streptococcus which had been in contact with normal rabbit serum instead of immune serum. When killed 18 hours later, rabbit 381 (immune serum prepared), streptococci were increased 20 times.

When the pleural cavity of an animal is, through proper preparation, at the point of complete destruction of the injected streptococci, an additional multiple of lethal doses may be reinjected and again sterilized.

Exper. 2.—Rabbit 376 was given 1 c.c. immune serum B and 18 hours later an additional 1 c.c. immune serum plus 200 M L D streptococcus. Three hours later, 200 additional lethal doses of the same culture were injected. The animal when examined 20 hours later gave sterile cultures from the pleural cavity.

This last experiment naturally suggests that the pleural fluid itself, under conditions which accompany sterilization of the cavity, might be expected to be endowed with bactericidal property. We have made numerous vain attempts to demonstrate any inherent bactericidal property in such pleural fluid. Such a fluid removed from a pleural cavity, and in which the bacteria have been enormously reduced or entirely eliminated, will not sterilize the usual multiples of the lethal dose freshly added when the mixture is injected into a normal pleural cavity. This may be logically explained on the supposition that in the prepared animal reserves of fresh cells are at call in the subpleural region and continue to arrive in the pleural cavity. This is further indicated in the necessity of both previous and simultaneous injection of immune serum to produce the best results.

Attempts to kill streptococci in vitro by means of pleural fluid have all been failures. Many variations in technic to accomplish this result have been tried. Fluids containing varying numbers of clasmatoocytes and polymorphonuclear cells have been tested; fluid before and after sterilization of streptococci in vivo have been used; the addition of more immune serum, or of alexin, or of both, has been tested; sensitized bacteria are also unaffected; injection of killed streptococci into the cavity a few hours before the fluid was used for test-tube experiment availed nothing. In all instances, the growth of the freshly added streptococci was more rapid in the pleural fluid mixture in the test-tube than in the broth controls.

DISCUSSION AND CONCLUSIONS

Rabbits may be protected against streptococcus empyema, produced by many multiples of the normally fatal dose, when an immune serum from rabbits is injected the day before infection and simultaneously with the streptococcus. Normal rabbit serum in the same doses also protects the majority of animals. The mechanism of the preventive effect differs in the two instances. The first injection of either serum produces the same increase of cells in the pleural cavity, and in both instances the relative proportions of clasmatoocytes, polymorphonuclears and lymphocytes are the same.

In the immune serum animals, sterilization of the infected cavity is rapid, being complete in 3 hours or less; this sterilization is accompanied by a critical rise in both clasmatoocytes and polymorphonuclear cells. In normal serum animals, there is a drop in the clasmatoocytes, and both clasmatoocytes and polymorphonuclears rise slowly to reach their respective maxima in about 12 hours. The clasmatoocytes never reach the level obtained in the immune serum animals, whereas the polymorphonuclears rise to twice the maximum of the immune series. Sterilization in the normal serum rabbits does not occur until the 2d or 3rd day.

These results, considered in the light of our previous report on the enhancing of natural resistance to streptococcus, still further indicate that the clasmatoocytes are the significant cells in experimental streptococcus infections.

We have been wholly unable to parallel in the test-tube the results obtained in the animal body. The pleural cavity of an immune serum prepared animal that has become sterile and is capable of still further destruction of streptococcus contains a fluid that has no manifest bactericidal properties under the conditions of our experiments.

MICROBIC RESPIRATION

III. RESPIRATION OF *TRYPANOSOMA LEWISI* AND *LEISHMANIA TROPICA**

From the Hygienic Laboratory of the University of Michigan, Ann Arbor

MALCOLM HERMAN SOULE

SYNOPSIS

Introduction.

Methods.

Tr. lewisi in Air; L. tropica in Air.

Fully Developed Cultures in Air.

L. tropica under Increased O₂ Tension; Tr. lewisi.

Tr. lewisi under Increased CO₂ Tension; L. tropica.

Tr. lewisi and L. tropica in the Absence of CO₂.

Tr. lewisi and L. tropica in the Absence of O₂.

Tr. lewisi and L. tropica on Serum Agar.

Tr. lewisi and L. tropica on Glucose Mediums.

Corrected Real Respiratory Quotients.

Summary.

INTRODUCTION

The pathogenic protozoa in a remarkably short time have risen from an obscure to a commanding position because of their importance in the causation of disease peculiar to the warm countries. The intermingling of troops from the whole world during the recent war gave evidence of increased infection from the known pathogenic forms. Of the many protozoa whose relation to disease have been proved, the hemoflagellates and the amebas are the most important.

In 1900, Laveran and Mesnil began their study of the trypanosomes, developing methods that led to the splendid series of researches which have come from the Pasteur Institute on these and related organisms.

Of no less importance to the protistologists was the cultivation on artificial mediums of Tr. lewisi by Novy and MacNeal¹ in 1903, who obtained, for the first time, strictly pure cultures of an animal parasite.

Since then a large number of flagellates have been grown on artificial mediums, the majority on either the original medium of these authors or on some modification of it. The serial cultivation, however, of such organisms as Tr. gambiense has not been attained.

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* A dissertation submitted in partial fulfillment of the requirements for the Degree of Doctor of Science in the University of Michigan.

¹ Contributions to Medical Research: Dedicated to V. C. Vaughan, Ann Arbor, Wahr, 1903, p. 549.

The vast literature of protozoa, which is growing and receiving continually new additions, is published in so many periodicals, some of them very difficult to obtain, that one hesitates and only ventures into this field with considerable trepidation.

It has been the impression among most workers that knowledge of the composition and reaction of the medium was most important. Little or no attention has been given the gaseous environment, and yet it is reasonable to believe that some recognition should be given to the respiratory changes.

Spallanzani² showed that all kinds of animals, whether they had lungs or not, gave off CO_2 , consumed O_2 and liberated heat, thus confirming Lavoisier's³ combustion theory of respiration.

By respiration in its widest sense must be understood all those processes in the organism whereby the potential energy stored up in chemical compounds of high complexity is set free to furnish the energy required by the organism for its vital activities. This object is effected by processes of oxidation; the result is the production of energy with the formation of simple chemical substances, such as water and CO_2 .

For the processes of oxidation, the organism either utilizes the free molecular O_2 from its air environment, or makes use of the oxygen which is present in organic combination in the nutritive material, as is the case with anaerobic organisms which live in a medium lacking free O_2 .

As the pathogenic hemoflagellates usually inhabit the blood stream, it is reasonable to believe that they would require some free O_2 for their development. It must be supposed that protozoa, like bacteria and other unicellular organisms, take up the required O_2 from the surrounding medium, and give off CO_2 .

The effect of various atmospheres has been tried on some of the lower forms of animal organisms. The experiments of Pütter⁴ on a number of ciliates, both free-living and parasitic, showed that when these animals were placed in an anaerobic environment different species reacted very differently to the conditions. Excess of O_2 was found by this author to have an injurious effect on *Spirostomum*. Jacobs⁵, placing drops of water containing *Paramecium* in an Engelmann gas chamber, observed CO_2 to be toxic for 12 varieties.

² Dissertazioni Varie, Vol. 2: Mémoire sulla Respirazione, 1826.

³ Histoire et Mémoires de l'Acad. d. Sc., Paris (1780); Mémoires, Année, 1777, p. 185.

⁴ Ztschr. f. allg. Physiol., 1904, 3, p. 363.

⁵ Jour. Exp. Zool., 1912, 12, pp. 519-542.

A quantitative study of the gas exchange by protozoa has been so beset with difficulties that few investigators have ventured into the field. It has been considered sufficient to detect respiration in a qualitative way, and for this two methods have been used. The first consists in passing measured quantities of air through the infusion with subsequent absorption by $\text{Ba}(\text{OH})_2$ of the CO_2 which is formed; the second, in observing changes in the color of indicator solutions which contain the organisms. The first method is truly qualitative in that no effort is made to measure the respiration of one particular species, and the second is obviously limited to the use of clear liquids on which many organisms do not grow. It is therefore readily understandable why no accurate measurements have been made.

It follows that quantitative information concerning the gaseous requirements of the protozoa is of importance. In the work which follows accurate estimations have been made, for the first time, of the respiratory changes induced by pure cultures of 2 species of pathogenic flagellates. Their growth has been measured by manometric observations, and the gas changes have been determined by exact quantitative analysis. For these organisms, the problem of the gas exchange has been satisfactorily solved. This work is one of a series of studies on microbial respiration which has been carried on in this laboratory for several years. The respiration of pure cultures of other protozoa will be taken up in subsequent papers.

Tr. lewisi, although not a strictly pathogenic form as found in the common rat, is an excellent type of the whole group. Owing to the almost universal distribution of its host, it has been found in all parts of the world. Since no difficulty is experienced in carrying the organism through a large series of subcultures, it lent itself very well to our study. The strain used in this work was isolated from an infected rat, and at present the culture is in its one hundred and twentieth generation.

L. tropica was first described by Cunningham⁶ (1885). In view of the fact that his description was imperfect, most authors give the credit of discovering the parasite to Wright⁷ (1903), who demonstrated it to be the cause of oriental sore. It is one of the strictly pathogenic protozoa which can be successfully cultivated and with

⁶ Hegner and Taliaferro: *Human Protozoology*, New York, Macmillan, 1924, p. 193.

⁷ *Jour. Med. Res.*, 1903, 10, p. 472.

ease. The strain used in this work was obtained from Dr. W. H. Brown of the Rockefeller Institute, and is now in the two hundred and thirteenth generation in this laboratory. We believe that the information concerning the gas requirements or respiratory changes of these two organisms will be of much value in the further study of other pathogenic forms.

METHODS

Culture Medium.—Finely comminuted choice lean beef was added to 2 parts of distilled water. The meat was extracted for 24 hours in the icebox, then strained, after which the liquid was heated to coagulate the protein and filtered.

To the meat extract, thus prepared, 1% of Witte's peptone and 0.5% of sodium chloride (Kahlbaum) were added. The liquid was then carefully adjusted to P_H 7.4 with NaOH; boiled, filtered, and 2% of agar added.

The agar medium was then filled into a number of 125 c. c. sterile Erlenmeyer flasks, and autoclaved at 110 C. for 20 minutes. For a given experiment, 1 or 2 of the flasks were liquefied by heat. As is known, the P_H of a medium changes on standing, and, since the stock medium was kept for some weeks, the P_H was again carefully adjusted just before being tubed. The medium was measured, by means of a standard pipet, into sterilized tubes, which were then autoclaved at 110 C. for 20 minutes; after this the tubes were cooled to about 50 C., and an equal volume of defibrinated rabbit blood or serum was added; the whole was then well mixed and slanted.

The blood was obtained by bleeding rabbits from the carotid using the technic of Novy;⁸ after defibrination, the blood was placed in the icebox for at least 2 days to avoid the germicidal effects, to which attention was called by Behrens.⁹

In order to insure sufficient inoculation, the transplantation was always made by means of a capillary pipet; 1 drop of the water of condensation being usually transferred and then spread over the surface of the medium with a sterile platinum wire to insure as even an inoculation as possible. Obviously, for purposes of comparison, it is desirable to inoculate approximately the same number of viable organisms. This, however, is seldom, if ever, realized, and the irregularities in manometric readings which may be noted in parallel tests find an explanation in the varying number of organisms at work, though the volume of air present is also a factor.

Culture Tubes.—An important consideration in the matter of the culture tube is the volume of the contained air. While it is possible to obtain manometric readings with small tubes, they are not suitable for the withdrawal of 10-20 c. c. of the gas for analysis. Hence, it was necessary to use the larger tubes described in Part I.

The plain tubes (20 x 200 mm.) of resistance glass were, at times, employed. When empty, the air capacity of such tubes ranged from 55 to 60 c. c. Shorter tubes, 20 x 150 mm., were necessary whenever the anaerobe jar was used.

The *h*-tube (fig. 1B, Part I) with an air volume of about 100 c. c. was usually used. The side tube permitted the introduction of alkali for absorption of CO₂, or of H₂O, or of any other desired reagent.

⁸ Laboratory Work in Bacteriology, Ann Arbor, Wahr, 1899, p. 460. Jour. Infect. Dis., 1917, 20, p. 502.

⁹ Jour. Infect. Dis., 1914, 15, pp. 24-62.

Side-arm tubes (fig. 2C, Part I) were found to be very useful, especially in experiments in which an all-glass seal was desired.

The inoculated *h*-tubes were attached to the slanted tip of manometers by means of selected rubber stoppers treated with glycerol, of No. 3 or 4 size. A dry stopper does not give a perfect contact with the glass, and as a result leakage occurs. The details of the preparation of the rubber stoppers will be found in Parts I and II.

Though convenient, the use of a rubber stopper in connection with gas work is not without objection, especially when the experiment is of long duration. An appreciable amount of CO₂ can be taken up by the rubber (Parts I and II). When utmost accuracy is desirable, it is preferable to have the culture tubes provided with ground glass caps (fig. 2B, Part I) which can be attached to the manometers by means of no. 25 rubber stoppers, or which can be fused on to the tip, thus giving an all-glass connection.

Culture Jars.—While the culture tubes when connected with manometers, as just described, were extremely useful in ascertaining the extent of gas exchange taking place under those conditions, they had the disadvantage of a limited capacity. This may be overcome in part by frequent evacuation and refilling with air or oxygen. For many purposes, however, it is preferable to make use of a large air chamber which will be gas-tight and which can be filled with air, or with varying percentages of O₂, CO₂, N₂, etc. The bottle or jar used for the cultivation of anaerobes is admirably adapted for this type of gas work.

Of the 2 types of Novy jars, the one with a special cock for vacuum work is best adapted for gas studies. The tail end of the cock can be connected with the manometer by means of a no. 25 rubber stopper, while the head is closed with a plugged stopper (fig. 6, Part I). The jar can be used without the manometer.

The organism to be tested was grown on the surface of plates, or in culture tubes, 20 x 150 mm. About 1-5 c.c. of distilled water were placed on the bottom of the jar, or in a tube, in order to provide quickly the necessary aqueous tension. The jar was then sealed in the manner described in Part I. With very little care, the jar can be made perfectly gas-tight. When filled with O₂ or CO₂, or with varying mixtures of gases, and kept at 37 C. for months, no loss in the gas content can be detected.

Refilling with Air or other Gases.—In the manipulative work incidental to the study of the gas changes in the culture tube, or in the anaerobe jar, it is necessary to be able to exhaust and to replace the gas contents with fresh air or with varying tensions of O₂, CO₂ or N₂. The full details of the procedure to be followed in such case will be found in Part I.

The Compensation Manometer.—In the study of gases, it is essential to be able to observe the pressure changes which take place within the culture tube, or jar, for only in this way is it possible to follow, hour by hour, or day by day, the reaction which takes place. The manometer not only reveals whether an organism is alive and growing, but also indicates the point when growth or respiration ceases. For the description of the manometer, and for details of its use with culture tubes or jars, reference is made to Part I.

Analyses.—The determination of the composition of the gas contained in the culture tube or jar, was made by means of Henderson's modification of the Haldane gas apparatus, and is fully described in Part I. At that place will be found, likewise, the method for the estimation of the CO₂ which is dissolved in the medium.

In the work which follows, it must be recognized that we are observing changes taking place in a definite confined quantity of gas, the initial composition of which is known. The manometric readings show the effect of the metabolic activities of the germs on this gaseous environment, hour by hour, or day by day, until respiration ceases. The analytical values are the quantitative expression of the changes produced by the organisms in this confined gas.

RESPIRATION OF *Tr. Lewisii* IN AIR

Exper. 1.—Nine *h*-tubes were carefully cleaned. They were first boiled in dilute alkali solution with soap and rinsed with distilled water; then boiled in dilute HCl and again repeatedly washed with water. Finally, they were allowed to dry. Loose cotton plugs were placed in the mouths of the tubes, and the tubes sterilized with dry heat at 200 C. On cooling, 5 c.c. of 2% agar were accurately measured into each of the tubes. They were then autoclaved at 110 C., 20 minutes, and allowed to cool in the autoclave to 50 C. Exactly 5 c.c. of sterile defibrinated rabbit blood were then added to each tube. The rabbit blood had been kept at 10 C. for 3 days. The tubes were thoroughly shaken to mix the medium, and then were inclined so that the length of the surface of the medium was about 11 cm. The tubes were thus slanted for 12 hours. This gave a soft, juicy medium which retained its shape unless placed in a vertical position.

Seven of the tubes were inoculated each with 1 drop of inoculum. This was spread over the entire surface of the medium with a sterile platinum wire. The 2 uninoculated tubes were similarly treated with sterile wire. Two drops of sterile distilled water were placed in the side-arm of each tube. This water furnished the moisture for the adjustment of the aqueous tension and insured an adequate supply for the growth of the germs.

The cotton plugs in the culture tubes were then cut off, and pushed within the tubes which were now attached to the manometers by means of rubber stoppers treated with glycerol. The stoppers were inserted as firmly as possible into the mouths of the tubes. The stopcocks of the manometers were carefully greased, probed and closed, after which rubber bands were placed around the handles of the cocks to hold them in their seats. The manometers, with the attached tubes, were placed in the hot-room at 29 C., the tubes being supported as shown in fig. 4, Part I.

The water in the side-arm was heated to about 70 C. with a free flame 4 times at intervals of 15 minutes, to adjust rapidly the aqueous tension. After allowing an additional hour for the apparatus to assume the temperature of the hot-room, the stopcocks were opened, and the mercury in the manometers was oscillated by means of a rubber bulb which was applied to cock 1. Cocks 1 and 3 were then closed, and the manometers read to be sure that they were properly equilibrated.

The manometers having been equilibrated, one of the uninoculated tubes was taken for analysis. This analysis was assumed to be representative of the composition of the atmosphere in all of the tubes. It might appear better to analyze each tube before equilibration. This, however, would necessitate the readmittance of air, which would cause a greater error than the assumed constancy of the atmosphere as found by the initial analysis of a control.

The intervals at which the manometers were read was based on the rate of growth, frequent observations being made during the period of rapid multiplication. It had been found that during the first 48 hours there was usually no change in the manometric readings. The gas samples during this interval showed

some, though relatively little, change in composition. The following results taken from an earlier experiment may serve as an example:

	Initial Analysis	24 hours	48 hours
CO ₂ per cent.	0.11	1.16	1.32
O ₂ per cent.	20.83	19.23	18.98
	20.94	20.39	20.30

This period in the growth of cultures is usually referred to as the "lag" period; its length depends on the activity of the germ. Thus, with the rapidly growing Hay bacillus this period is, as a rule, not over 2 hours in length. Sherman and Albus¹⁰ view this latent period as a biologic rejuvenescence, which,

TABLE 1
MANOMETRIC READINGS AND ANALYSES OF GROWTH OF TR. LEWISI IN AIR

Tube No.	Cultures							Uninoculated	
								Initial Control	Final Control
Hrs.	1	2	3	4	5	6	7	8	9
0.....	Mm. 0	Mm. 0	Mm. 0	Mm. 0	Mm. 0	Mm. 0	Mm. 0	Mm. 0	Mm. 0
2.....	0	0	0	0	0	0	0	0
24.....	0	0	0	0	0	0	0	0
48.....	0	0	0	0	0	0	0	0
72.....	-1	0	0	0	-1	0	0	0
96.....	-3*	-2	-1	-3	3	-1	-2	0
120.....	6	4	3	9	3	5	-1
144.....	-13*	12	12	13	9	11	2
168.....	22	20	19	19	22	3
192.....	34	28	28	29	27	4
216.....	-34*	39	35	37	36	4
240.....	48	46	45	44	4
264.....	-51*	48	45	44	4
288.....	-53†	46	46	4
312.....	48	46	6
336.....	-50†	-46†	-8
Analyses at end of...	96 Hrs.	144 Hrs.	216 Hrs.	264 Hrs.	288 Hrs.	336 Hrs.	336 Hrs.	0 Hrs.	336 Hrs.
CO ₂	2.04	4.71	10.08	13.46	14.05	14.99	15.05	0.21	1.43
O ₂	18.60	14.70	7.11	0.98	0.06	0.00	0.00	20.77	18.39
Total.....	20.64	19.41	17.19	14.44	14.11	14.99	15.05	20.98	19.82

The manometers were equilibrated; barometer, 748 mm.; temperature, 29 C.

* The cultures were very rich.

† The organisms were granulated.

however, is not the generally accepted interpretation. Some workers prefer to consider it as an expression of an "injury" received by the organism in its previous environment. Wherry and Ervin¹¹ refer to the interval as a latent period during which CO₂ accumulates to an optimal concentration.

The manometers were read at intervals of 24 hours. Before reading they were given a sharp jerk so as to cause oscillation of the mercury.

¹⁰ Jour. Bacteriol., 1924, 9, p. 303.

¹¹ Jour. Infect. Dis., 1918, 22, p. 194.

Table 1 shows the manometric readings and the analyses of tubes inoculated with *Tr. lewisi*. It is to be noted that the period of the tests varied from 4 to 14 days. After the analyses, the tubes were removed from the manometers and the growth examined microscopically.

The differences in the rate of development of the negative pressure was due, in part, to the variation in the number of organisms planted. It was obviously impossible to get exact duplicate seedings. Moreover, the capacity of the tubes varied somewhat, and the negative pressure would naturally develop more rapidly in the smaller tubes. The maximal variation in the size of the tubes used in this experiment was 7 c.c.

Before proceeding to discuss the analytical data, the microscopic examinations should be considered. It will be noted that the cultures were exceedingly rich in motile organisms up to the end of the 264th hour. In the next 48 hours, however, marked degeneration took place.

The initial gas content of the tubes was not that of pure air, as shown by the control analysis. The increase in the CO_2 over that usually found in air was due probably to the liberation of dissolved CO_2 from the medium.

The rapid development of CO_2 and the accompanying loss of oxygen, beginning at about the 96th hour, was due to the vigorous multiplication of the culture. The rapid respiration continued until the concentration of oxygen had become considerably reduced.

After the disappearance of the oxygen (288th hour), the CO_2 continued to increase. This increase, slow as it is, seemed to go on indefinitely. This continued production of CO_2 in the culture was paralleled by like changes in the uninoculated control tubes. The reaction was clearly a secondary phenomenon due to auto-oxidation and possibly to a progressive decarboxylation of the protein cleavage products. In experiments extending over a period of 6 months, no end to the CO_2 production was observed.

It is extremely interesting to compare our manometric observations with the course of infection in rats. A rat inoculated with *Tr. lewisi* will show an incubation period of 4 days. The parasites then undergo rapid production for about 6 days, after which the rate of reproduction is retarded, and finally it is inhibited by the 10th day. This inhibition, according to Taliaferro,¹² is due to the formation of a reaction product in the serum which, however, does not kill the organisms.

¹² Footnote 6, p. 139.

Manometrically, the cultures have a "lag" period of 4 days, then rapid reproduction takes place for about 6 days, at which time further growth is inhibited by the lack of oxygen. That the absence of oxygen in the tube is the cause of this inhibition will be shown in exper. 4, in which active growth followed aeration of the culture.

Under the latter conditions, growth, however, does not continue indefinitely. It eventually is arrested though an abundance of oxygen is present. The accumulation of products, whether made directly by the organism or by secondary changes in the medium, brings about cessation of growth and degeneration.

TABLE 1a
CALCULATED RESPIRATORY QUOTIENTS AND MANOMETRIC READINGS FOR EXPER. 1

Tube No.	1	2	3	4	5	6	7
Analyses CO ₂	2.04	4.71	10.08	13.46	14.05	14.99	15.05
O ₂	18.60	14.70	7.11	0.98	0.06	0.00	0.00
N ₂	20.64	19.41	17.19	14.44	14.11	14.99	15.05
	79.36	80.59	82.81	85.56	85.89	85.01	84.95
Total.....	100.00	100.00	100.00	100.00	100.00	100.00	100.00
Corrected analyses CO ₂	2.03	4.61	9.62	12.27	12.92	13.93	13.99
O ₂	18.54	14.41	6.78	0.89	0.05	0.00	0.00
N ₂	20.57	19.02	16.40	13.16	12.97	13.93	13.99
	79.02	79.03	79.02	79.02	79.03	79.02	79.03
Total.....	99.59	98.05	95.42	91.18	92.00	92.95	93.02
Real gain CO ₂	1.82	4.40	9.41	12.06	12.71	12.72	13.78
Real loss O ₂	2.23	6.36	13.99	19.88	20.72	20.77	20.77
Real resp. quot.	0.816	0.691	0.672	0.606	0.613	0.612	0.663
Calc. manometric readings..	-2.88	-14.07	-32.88	-56.14	-57.51	-49.90	-50.18
Corr. obs. man. readings....	-3.04	-13.36	-34.6	-52.0	-53.8	-50.7	-46.7

Corrected analyses = analytical value \times nitrogen factor, i. e., for tube 1 factor = $\frac{79.02}{79.36}$

Calculated manometric readings = real loss \times (B - aqueous tension)

We may now consider the uninoculated final control. It remained sterile throughout the experiment. It is worthy of note that this tube showed a slowly progressive rise in negative pressure. Analysis revealed an increase in the CO₂ content and a loss of O₂. The negative pressure was due, in the first place, to the auto-oxidation of the medium, and, secondly, to the solution of CO₂ by the medium and by the rubber stopper. We were unable to determine the consideration to be given this control in our calculations, and for that reason no deductions were made.

The respiratory quotients, as calculated from the analyses, assuming the initial control to be representative for all of the tubes, are given in table 1a.

It is to be noted that the calculated respiratory quotients are low and that they show some variation. These differences, obtained under presumably identical conditions, indicate loss of CO_2 by solution in the rubber stopper, and even in the medium. That this is true will be demonstrated later when the jar method of determining the respiratory quotient is taken up.

The corrected observed manometric readings were, as a rule, in close concordance with the calculated manometric readings based on the analyses. The greatest difference was that given by tube 4, the calculated reading being 56.1 mm., while the corrected observed reading was 52 mm.

It was assumed, in calculating the readings from analyses, that only O_2 and CO_2 underwent a change in concentration. The liberation of small quantities of such substances as N_2 , NH_3 or other products exerting a vapor pressure would influence the observed readings but would not be accounted for in the calculated values.

RESPIRATION OF *L. TROPICA* IN AIR

The growth of *L. tropica* on blood agar was such as to be observable macroscopically. On the second day after inoculation, dark round areas appeared on the surface of the medium and seemed to extend quite deep. These spots increased in size until the entire slant had lost its rich red hemoglobin color, which gave way to the formation of the dark brown hematin. At this stage, the surface was covered with a thick, mucus-like, moist, slimy growth, very much like that of a bacterial culture. This surface growth was prominent only when large culture tubes were used. It was due, therefore, to the presence of a relatively large quantity of oxygen.

In the 2 experiments which follow we were able to observe the growth of *L. tropica* over a period of 240 hours. These experiments were performed more than a year apart, and hence cover two different periods in the life of the culture. In exper. 2, the manometers were read at 12 hour intervals, and the tubes were analyzed at 24 hour intervals, beginning with the 24th hour. In exper. 3, the observations were made at 24 hour intervals, and the first analysis was made at the end of the 96th hour.

Exper. 2.—Seven *h*-tubes were prepared as already explained. Each of 5 of the tubes received 1 drop of inoculum, which was then spread over the entire surface of the medium with a sterile platinum wire. The 2 uninoculated tubes were treated, in like manner, with the sterile wire in order to have the same exposure to air. Two drops of sterile distilled water were placed in the side-arm of each tube. This water was not heated as we did not appreciate, at that time, the problem of aqueous tension. This water was intended to supply the moisture for the growth of the germ and the needed aqueous tension.

As in the previous experiment, the cotton plugs on the culture tubes were cut off, and the tubes were then attached to manometers using the same procedure as in exper. 1.

After allowing 2 hours for the apparatus to assume the temperature of the hot-room, 29 C., the manometers were equilibrated in the manner already given. As before, an initial control analysis was made, and this was assumed to be representative of the composition of the atmosphere in all of the tubes.

Exper. 3.—Nine *h*-tubes were prepared as before. The preparation of the medium, the inoculation, and the connection of the tubes with the manometers was carried out exactly as in exper. 1. In this experiment, however, the water in the side-arms was heated in order to adjust quickly the aqueous tension. The manometers were equilibrated, after being in the hot-room for 2 hours, and then one of the uninoculated tubes was analyzed as an initial control.

The manometric readings and analyses for these 2 experiments are given in tables 2 and 3.

The difference in the rate of development of the negative pressures in these 2 experiments should be noted. A comparison of the readings in these 2 tables shows that the culture in exper. 3 was the more active. The manometric readings indicated a consistent rate of growth in the tubes of each experiment, but there was a decided difference in the values given by the two experiments.

This difference is not readily accounted for, but the explanation probably lies in 3 factors, namely, the viability of the germs planted, the absolute number of such organisms inoculated and a possible variation in the mediums used. Certainly, the results in table 3 at the end of 96 hours show much greater activity than is seen at the same time in table 2.

The microscopic examination of each tube, made after sampling, was very interesting. In exper. 2, the organisms at the end of 24 hours were not conspicuously active, but they had a healthy appearance.

The other tubes examined after 48, 72, 96 and 120 hours were exceedingly rich with masses of perfect flagellates, showing no indications of unfavorable conditions. In exper. 3, there was the same richness of growth in tubes 1, 2 and 3. The next tube examined (168 hours) showed a marked difference; the organisms were granulated and slow. Examinations of tubes 5, 6, 7 (192, 216 and 240 hours) showed extreme granulation of the germs with only now and then a motile cell. A study of the analyses reveals the fact that the O_2 was practically gone at the end of 144 hours and that the organisms were subjected to the action of about 14% of CO_2 .

It was observed in all of these test-tube experiments that the cultures were rich and that the organisms were extremely active, though the tension of CO_2 was greatly increased, while that of O_2 was considerably decreased as compared with the tension of these gases in the blood.

TABLE 2
MANOMETRIC READINGS AND ANALYSES OF GROWTH OF *L. TROPICA* IN AIR

	Cultures					Uninoculated	
						Final Control	Initial Control
Tube No.	1	2	3	4	5	6	7
Hrs.							
0.....	0	0	0	0	0	0	0
12.....	0	0	0	0	0	0
24.....	-2*	-1	-4	-4	-2	-1
36.....	4	6	5	7	1
48.....	-9*	9	11	18	2
72.....	-22*	23	28	2
96.....	-28*	39	2
120.....	-39*	-4
Analyses at end of.....	24 Hrs.	48 Hrs.	72 Hrs.	96 Hrs.	120 Hrs.	120 Hrs.	0 Hrs.
CO ₂	1.89	4.03	4.69	8.33	10.70	1.67	0.14
O ₂	18.76	15.23	14.36	8.86	5.22	19.12	20.80
Total.....	20.65	19.26	19.05	17.19	15.92	20.79	20.94

The manometers were equilibrated at a barometric pressure of 756 mm., temperature 30.5 C.
* The cultures were very rich.

TABLE 2a
CALCULATED RESPIRATORY QUOTIENTS AND MANOMETRIC READINGS FOR EXPER. 2

Tube No.	1	2	3	4	5
Analyses CO ₂	1.89	4.03	4.69	8.33	10.70
O ₂	18.76	15.23	14.36	8.86	5.22
N ₂	20.65	19.26	19.05	17.19	15.92
Total.....	79.35	80.74	80.95	82.81	84.08
	100.00	100.00	100.00	100.00	100.00
Corr. analyses CO ₂	1.88	3.94	4.58	7.95	10.00
O ₂	18.69	14.91	14.02	8.45	4.90
N ₂	20.57	18.85	18.60	16.40	14.90
Total.....	79.06	79.06	79.06	79.06	79.06
	99.63	97.91	97.66	95.46	94.02
Real gain CO ₂	1.74	3.80	4.44	7.81	9.92
Real loss O ₂	2.11	5.89	5.78	11.55	15.90
Respiratory quotient.....	0.824	0.645	0.768	0.676	0.624
Calc. man. reading.....	-2.67	-15.18	-15.9	-32.8	-43.24
Corr. obs. man. reading.....	-2.03	-9.15	-22.35	-28.7	-39.66

Corrected analyses = analytical value × nitrogen factor.
Calculated manometric reading = real loss × (B - aqueous tension).

TABLE 3
MANOMETRIC READINGS AND ANALYSES OF GROWTH OF *L. TROPICA* IN AIR

Tube No.	Cultures							Uninoculated	
								Initial Control	Final Control
Hrs.	1	2	3	4	5	6	7	8	9
0.....	Mm. 0	Mm. 0	Mm. 0	Mm. 0	Mm. 0	Mm. 0	Mm. 0	Mm. 0	Mm. 0
24.....	0	0	0	0	0	0	0	0
48.....	-10	-10	-3	-5	-11	-9	-3	0
72.....	31	24	16	15	20	28	15	0
96.....	-47*	38	33	27	32	32	26	0
120.....	-49*	46	39	41	42	37	0
144.....	-52*	51	45	49	47	-1
168.....	-55†	53	49	48	2
192.....	-53†	51	46	3
216.....	-51†	46	4
240.....	-46†	4
336.....	-8
Analyses at end of...	96 Hrs.	120 Hrs.	144 Hrs.	168 Hrs.	192 Hrs.	216 Hrs.	240 Hrs.	0 Hrs.	336 Hrs.
CO ₂	11.52	10.41	14.70	13.16	13.91	14.15	15.33	0.21	1.43
O ₂	4.08	5.87	0.00	1.51	1.08	1.03	0.00	20.76	18.39
Total.....	15.60	16.28	14.70	14.67	14.99	15.18	15.33	20.97	19.82

The manometers were equilibrated, the barometric pressure being 748 mm., and the temperature 29.8 C.

* The cultures were very rich.

† The organisms were nearly or completely granulated.

TABLE 3a
CALCULATED RESPIRATORY QUOTIENTS AND MANOMETRIC READINGS FOR EXPER. 3

Tube No.	1	2	3	4	5	6	7
Analyses CO ₂	11.52	10.42	14.70	13.16	13.91	14.15	15.33
O ₂	4.08	5.87	0.00	1.51	1.08	1.03	0.00
N ₂	15.60	16.29	14.70	14.67	14.99	15.18	15.33
Total.....	84.40	83.71	85.30	85.33	85.01	84.82	84.67
Corrected analyses CO ₂	10.78	9.83	13.61	12.19	12.93	13.18	14.31
O ₂	3.80	5.54	0.00	1.40	1.03	0.96	0.00
N ₂	14.58	15.37	13.61	13.59	13.96	14.14	14.31
Total.....	79.05	79.03	79.04	79.02	79.00	79.03	79.03
Real gain CO ₂	10.57	9.62	13.40	11.98	12.72	12.97	13.35
Real loss O ₂	16.96	15.22	20.76	19.36	19.73	19.80	20.76
Resp. quotient.....	0.620	0.632	0.645	0.614	0.644	0.655	0.643
Calc. man. readings.....	-45.79	-40.19	-52.7	-52.8	-50.22	-48.9	-54.56
Corr. obs. man. readings....	-47.51	-49.6	-52.7	-55.88	-53.9	-51.46	-46.6

Corrected analyses = analytical value × nitrogen factor.

Calculated manometric readings = real loss × (B - aqueous tension)

A comparison of tables 1 and 3 shows a striking difference in the rate of growth of the 2 organisms. These 2 tables lend themselves very well to comparison, since blood from the same animal was used in the preparation of the mediums. In time, both organisms consumed all of the oxygen. This was accomplished by *L. tropica* in one-half of the time required by *Tr. lewisi*. The more rapid and more luxuriant growth of the former accounted for this difference.

Tables 2*a* and 3*a* show the calculated respiratory quotients and calculated manometric readings for the cultures just described. The quotients in table 2*a* vary somewhat, from 0.624 to 0.824, while in table 3*a* the differences are not so great (0.614-0.655), though the values are low for the reasons mentioned in connection with table 1*a*.

A comparison of the calculated and observed manometric readings, as given in either table 2*a* or 3*a*, shows more or less variation. This is due in part to the difficulty of securing an exact control analysis of the air which is present in each tube at the start of the experiment. In addition to this, if the aqueous tension is not fully established at the time of equilibration, it will result in a lower observed reading than is deduced from the analysis.

It was assumed in the early part of this work that when water was placed in the side-arm, the aqueous tension would adjust itself during the 2 hours previous to equilibration. This, however, is not the case, for a positive pressure of 1-5 mm. was often observed after equilibration, clearly due to the gradual adjustment of vapor pressure. It therefore became evident that the adjustment of aqueous tension could not be secured in this way. Hence, in connection with the tests given in table 3*a*, an effort was made to obviate this by heating the water contained in the side-arm. This, it will be seen, resulted in manometric readings which, with one exception, were somewhat higher than those calculated.

The same treatment applied to the tubes given in table 1*a* produced fairly concordant results, the variation being less than 4 mm.

FULLY DEVELOPED CULTURES OF *TR. LEWISI* AND *L. TROPICA* IN AIR

At the end of the "lag" period in the previous experiments, as indicated, the germs were undergoing very rapid reproduction. It is evident that observations made during this interval necessarily represent changes due to organisms which are undergoing little or no multiplication. The analytical values obtained during the period of active reproduction which continues until the maximal negative pressure is

reached, are representative of a young and rapidly growing culture. It is unlikely that the respiratory quotient during this interval is different from that at any other time. The following experiment was intended to bring out the changes produced by cultures which were fully developed and undergoing rapid multiplication. On account of the large number of organisms present, it was expected that the respiration process would begin and end earlier than it did when the tubes were freshly inoculated.

TABLE 4
SHOWING, IN THE FIRST AERATION TEST, THE RAPIDITY IN RISE OF NEGATIVE PRESSURE WITH THE FINAL RESULTS OF ANALYSES

Tube 4, Exper. 3 L. tropica		Tube 4, Exper. 1 Tr. Lewisi	
Hrs.	Mm.	Hrs.	Mm.
0.....	0	0.....	0
4.....	0	2.....	0
8.....	-1	7.....	0
18.....	10	9.....	-1
23.....	12	20.....	7
43.....	25	28.....	10
49.....	28	33.....	26
54.....	30	45.....	24
56.....	31	57.....	30
66.....	38	70.....	40
74.....	39	78.....	44
90.....	46	97.....	51
102.....	49	117.....	57
114.....	56	141.....	-64
138.....	62		
162.....	-62		
Then analyzed they gave % CO ₂		12.96	
O ₂		0.10	
		13.06	
The same tubes in the initial test gave a maximal pressure of.....		-51	
In 168 Hrs.		264 Hrs.	
And analysis gave CO ₂		13.46	
O ₂		6.98	
		14.44	

Exper. 4.—Several of the tubes from exper. 1 and 3 were taken. The tubes after analysis were removed from the manometers, and a specimen was taken with a platinum loop for microscopic examination. The cotton plugs were put loosely in the tubes which were then left to aerate by diffusion. The bright red hemoglobin color returned to the medium as the CO₂ traveled outward. The color in the Tr. lewisi tubes became as bright as when originally planted; the L. tropica tubes seemed to be darker as though some permanent reaction had taken place in the medium. After 24 hours of diffusion, to make sure that all of the gas in the culture tubes was replaced with normal air, a glass tube was introduced, and filtered air was blown through for 5 minutes. The side-arms were also thoroughly aerated. This method of aeration was employed in this experiment because it had been necessary to remove the tubes from the manometers for the microscopic examination.

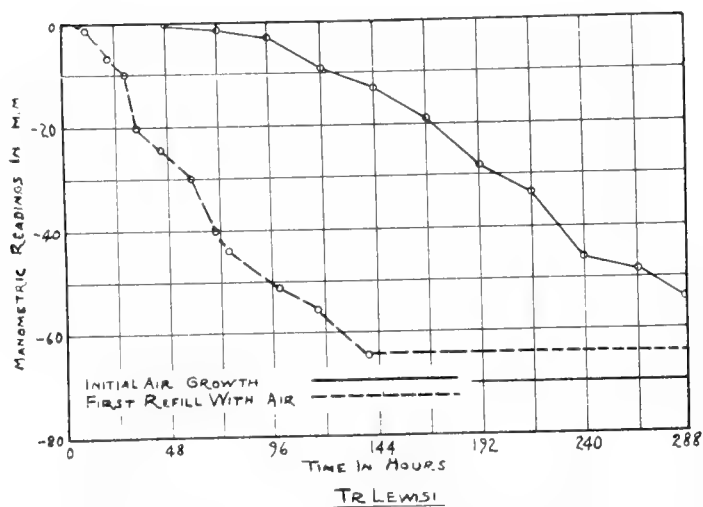


Chart 1.—Showing the rapidity in development of negative pressure of an initial culture and the same culture in the first aeration. *Tr. lewisi*.

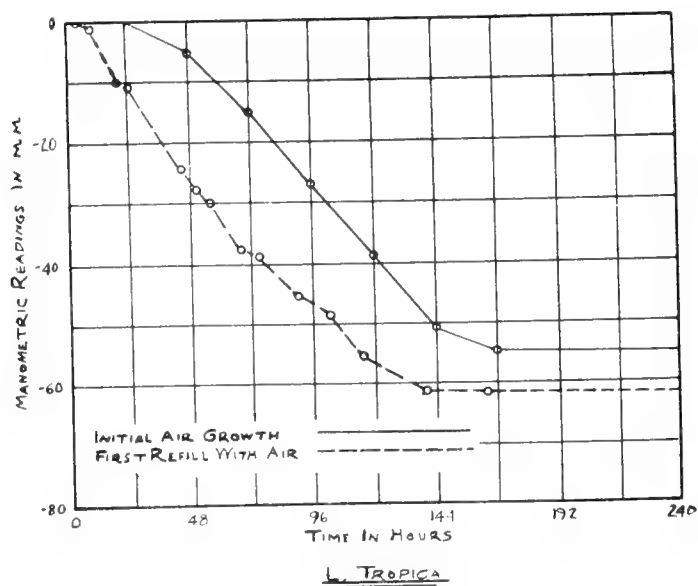


Chart 2.—Showing the rapidity in development of negative pressure of an initial culture and the same culture in the first aeration. *L. tropica*.

The tubes were then reattached to the manometers in the usual manner, returned to 29 C., and after an interval of 2 hours, they were equilibrated, and cocks 1 and 3 were closed. The aeration of the culture tubes was effected without the slightest evidence of contamination.

The results of this experiment for 1 tube each of *L. tropica* and *Tr. lewisi* are given in table 4, and are also presented graphically in charts 1 and 2. The culture of the former, as indicated in table 3, was highly granular, while that of the latter was very rich in good cells (table 1).

It should be pointed out that the yield of CO_2 in these tests was lower than that obtained in the first trials. This was probably due to some fixation of O_2 by the reducing products present in the more or less disintegrating mass of cells.

It will be seen that the negative pressure manifested itself earlier than at the start of the other experiments. Likewise, it will be seen that the previous maximum was reached in a shorter time.

The manometric response, as seen in table 4, indicated that a distinct "lag" period was present, although it was shorter than in exper. 1 and 2. It was possibly due to the age and condition of the cultures employed. This was contrary to what might be expected with an active and well developed culture. Consequently, the following experiment was made to show the absence of such a "lag" period in a young and well developed culture of *L. tropica*.

Exper. 5.—Four side-arm tubes (fig. 2C, Part I) were carefully cleaned and dried. Tight cotton plugs were placed in the mouths of the tubes and a loose pledget in the side-arm. The tubes were sterilized at 200 C. On cooling, 5 c.c. of 2% agar were measured into each tube. They were then autoclaved at 110 C. for 20 minutes; cooled in the autoclave to 50 C., after which 5 c.c. of sterile defibrinated rabbit blood were added to each tube. The rabbit blood had been kept at 10 C. for 48 hours. The tubes were shaken and slanted so that the length of the surface of the medium was about 10 cm. The tubes were left in this position for 12 hours.

Two of the tubes were inoculated as usual. The 2 control tubes were also treated with the sterile platinum wire. The mouth of each tube, after removal of the cotton plug, was now sealed in the blast lamp, and then each tube was sealed to the end of a manometer, thus avoiding all rubber connections. The actual air capacity of these tubes was thus reduced to about 30 c.c. The manometers were placed at 29 C., and equilibrated after 2 hours. One of the uninoculated tubes (No. 3) was then analyzed as the initial control.

The culture tubes were analyzed at the end of 95 hours when it seemed certain that the maximal negative pressure had been reached. The manometers with the 2 inoculated tubes were then attached by means of cocks 3 to a glass rake, which connected with the vacuumeter, and the tubes were evacuated to -715 mm., after which air was admitted through a tube filled with sterile cotton.

This operation was repeated 5 times so as to replace all the gas in the culture tubes with normal air. The manometers were then equilibrated, and cocks 1 and 3 were closed. Tube 1 was analyzed as an initial control. The results of this experiment are given in table 5.

It will be seen that in tube 2, after aeration, the negative pressure began to appear at once, and that the maximum was reached in a somewhat shorter time than before. Thus, it was evident that no

TABLE 5
SHOWING, IN THE FIRST AERATION TEST, THE ABSENCE OF A "LAG" PERIOD. L. TROPICA

Tube No.	Cultures		Uninoculated	
	1	2	Initial Control	Final Control
			3	4
Hrs.	Mm.	Mm.	Mm.	Mm.
0*	0	0	0	0
15.....	-2	0	0
29.....	3	0	0
41.....	8	-4	0
53.....	19	17	0
65.....	33	39	0
77.....	47	57	-1
89.....	57	59	2
95.....	-57	-59	-2
First aeration				
0†.....	0	0		
1.....	-1		
2.....	2		
3.....	3		
4.....	4		
5.....	5		
6.....	6		
7.....	7		
8.....	8		
14.....	14		
26.....	29		
38.....	42		
52.....	51		
62.....	-54		

* The manometers were equilibrated, the barometer at 750 mm., temperature 29 C.
† The manometers were equilibrated, the barometer at 748 mm., temperature 29 C.

"lag" period was present when a fully developed, active culture was employed. It should be added that this culture, at the time of aeration, was presumably very rich and in good condition, whereas in the older culture of L. tropica which was used in exper. 4, the organisms were nearly or completely granulated as indicated in table 3.

L. TROPICA UNDER INCREASED OXYGEN TENSION

Shortly after the isolation of oxygen, observers found that respiration of the gas produced inflammatory effects. No appreciable change

in the elimination of CO_2 was observed by Regnault and Reiset.¹³ Later, Lorrain Smith¹⁴ found that mice subjected to partial pressures of 73.6-79.9% of oxygen succumbed in 3-4 days. More recently, Moore and Williams¹⁵ found that the tubercle bacillus grew badly or not at all in concentrations of about 80% of an atmosphere of oxygen. Adams,¹⁶ extending the work of these 2 investigators, found only 2 organisms out of 26 tested to be oxyphobic, the tubercle bacillus and *B. pestis*. Concentrations of 60% or more were toxic for the plague bacillus, but only inhibitive for the tubercle bacillus. He also studied the effect of high oxygen tensions on mammals.

The use of O_2 in the treatment of disease makes the study of its effects on the pathogenic organisms of special importance. It might be assumed that parasitic organisms are adapted to low tensions of CO_2 and O_2 , such as exist in the tissues, and that an appreciable change in the concentration of these gases in the culture tubes would inhibit their growth. It was therefore with the object of determining the influence of high tensions of O_2 that the following experiments were made.

It should be pointed out before proceeding with the experimental work, that when the germs are planted on a solid medium, it does not follow that they will all remain on such surface. It is possible, especially with a fluid inoculum and a soft medium, for some of the organisms to penetrate along the glass wall, and even through the medium itself, into the deeper layers. Such organisms, sheltered from the direct effect of the higher concentrations, may proceed to prepare conditions which are favorable to those remaining on the surface. Irregularity in the manometric readings, in parallel tubes, as seen in tables 12 and 13, may be due to this factor.

The manipulative work necessary in connection with the introduction of different gas tensions into culture tubes or jars has been given in detail in Part I. Consequently, only an outline of the procedure is given below.

In each of the experiments which follow, 2 control tubes were inoculated at the same time, closed with rubber caps and incubated at the same temperature as the others. At the end of 4 or 5 days, they were examined. In every instance, a rich growth was obtained thus eliminating any question as to the viability of the germs planted.

¹³ Ann. d. Chim. et d. Phys. (3), 1849, 26, p. 299.

¹⁴ Jour. Physiol., 1899, 24, p. 19.

¹⁵ Bio-Chem. Jour., 1909, 4, p. 177; 1911, 5, p. 181.

¹⁶ Ibid., 1912, 6, p. 297.

Growth in 30% Oxygen; Exper. 6.—Four *h*-tubes were prepared in the usual way. Two of the tubes were inoculated with 1 drop of the rich culture fluid, and the inoculum was subsequently spread with a sterile platinum wire. The 2 uninoculated tubes were also treated with the sterile wire. The loose cotton plugs were cut off and pushed into the tubes, which were then attached to the manometers by means of rubber stoppers treated with glycerol. The manometers were then connected by cocks 3 to a glass rake, which in turn was joined to the special T connector shown in fig. 8, Part I. The latter was attached to the suction apparatus and to the O₂ and N₂ supply.

With the necessary precaution as to the manometers, the tubes were evacuated to —700 mm., and then N₂ was admitted.

This operation was repeated 5 times, allowing 10 minutes between exhaustions for diffusion of the gases. Finally, the tubes were evacuated to —225 mm., and, after replacing the N₂ in the connecting line with O₂, the latter was admitted into the tubes until the mercury in the manometers indicated the zero level. The no. 3 cocks were then closed and the manometers disconnected from the rake and placed in the hot-room. After 2 hours, they were equilibrated with the tip of stop-cock 3 below the level of water in a small beaker.

After equilibration, one of the tubes was analyzed and was found to have 31.7% of O₂.

The reason why a higher value was obtained than was contemplated was due to the fact that the uncorrected barometric pressure was used in the calculation. The barometric pressure was 744 mm., and since the aqueous tension at 31 C. is 33.7 mm., it follows that $B - T = 710.3$, and 30% of this equals 213, whereas actually the evacuation was carried to —223 mm.

It should be mentioned here that in all of the work in which the gas to be determined was more than 25% of the sample, it was necessary to take less than 3 c.c., for the reason that on the gas buret, though of 10 c.c. capacity, only the lower 3 c.c. were graduated. To obtain this small sample, it was necessary first to measure out approximately 7 c.c. of nitrogen, which was then transferred into the pyrogallate tube. About 3 c.c. of the sample were then drawn into the buret, the nitrogen was then brought over, and the total volume read.

The limit of accuracy therefore in these experiments was less than when a 10 c.c. sample was used. Extreme instances of such error can be seen in the control tubes in tables 6 and 7.

It will be seen from table 6, which contains the results of this experiment, that a pressure of —96 mm. was reached in about 192 hours, and that the analyses showed the O₂ to be reduced to 0.13 and 0.91%, while the CO₂ content rose to more than 20%.

The increase of oxygen tension to 30% seemed to have a stimulative effect on the growth of the organism, as indicated by the rapid fall in the pressure. The maximal negative pressure was obtained when the O₂ was nearly gone. This point was reached in 192 hours, or in about the same time as when air was present (table 3). It is to be noted that while, in the latter, the maximal pressure was about —53 mm., in this experiment, it rose to —96 mm. In tables 7 and 8, the pressure became considerably higher. This increase in the negative pressure is a necessary consequence of an increase in the amount of O₂ consumed, as has been pointed out in Part I.

Growth in 40% O₂; Exper. 7.—The procedure in this experiment was the same as in the preceding one, except that a higher concentration of O₂ was used. The results will be found in table 7. It is worthy of note that the negative

TABLE 6
MANOMETRIC READINGS AND ANALYSES OF GROWTH OF L. TROPICA IN 30% OXYGEN

Tube No.	Cultures		Uninoculated	
			Final Control	Initial Control
Hrs.	1	2	3	4
0.....	Mm. 0	Mm. 0	Mm. 0	Mm. 0
24.....	-2	-2	0
48.....	22	8	0
72.....	41	23	-1
96.....	57	40	2
120.....	74	56	2
144.....	85	70	2
168.....	96	84	2
192.....	-97*	95	2
216.....	-96*	-2
Analyses at end of.....	192 Hrs.	216 Hrs.	216 Hrs.	0 Hrs.
CO ₂	21.38	20.27	0.79	0.15
O ₂	0.13	0.91	26.47	31.47
	21.51	21.18	27.26	31.62

The manometers were equilibrated with the barometer reading 744 mm. and the temperature 31 C.
* The organisms were granulated.

TABLE 7
MANOMETRIC READINGS AND ANALYSES OF GROWTH OF L. TROPICA IN 40% OXYGEN

Tube No.	Cultures		Uninoculated	
			Final Control	Initial Control
Hrs.	1	2	3	4
0.....	Mm. 0	Mm. 0	Mm. 0	Mm. 0
24.....	-4	-2	0
48.....	11	18	0
72.....	29	43	-2
96.....	45	64	2
120.....	57	78	2
144.....	68	92	2
168.....	79	104	2
192.....	88	113	2
216.....	110	117	2
240.....	-110*	-117*	-2
Analyses at end of.....	240 Hrs.	240 Hrs.	240 Hrs.	0 Hrs.
CO ₂	25.99	26.18	0.85	0.63
O ₂	3.28	0.00	41.72	37.94
Total.....	29.27	26.18	42.57	38.67

The manometers were equilibrated at a barometric pressure of 744 mm. and a temperature of 31 C.
* The organisms were granulated.

pressure reached was higher than when 30% O₂ was used, being —110 and —117 mm., respectively. Analysis showed the presence of 26% CO₂, and in one tube (no. 2) an entire absence of O₂. Clearly, this higher content of O₂ stimulates rather than retards the growth of this organism.

Growth in 50% O₂; Exper. 8.—The manometric readings and analytical data are given in table 8. The growth of the organisms in this atmosphere was luxuriant. Macroscopic examination showed a heavy surface growth. The ability of the germ to grow in this concentration of O₂ and produce such a high partial pressure of CO₂ was extremely interesting, since, as will be shown later the use of 30% of CO₂ does not permit the growth of the organism in a freshly inoculated tube.

TABLE 8
MANOMETRIC READINGS AND ANALYSES OF GROWTH OF *L. TROPICA* IN 50% OXYGEN

Tube No.	Cultures		Uninoculated	
			Final Control	Initial Control
	1	2	3	4
Hrs.	Mm.	Mm.	Mm.	Mm.
0.	0	0	0	0
24.	—3	—3	0
48.	15	16	0
72.	40	30	0
96.	65	51	0
120.	91	67	—2
144.	108	76	2
168.	126	89	2
192.	143	98	2
216.	152	102	2
240.	154	111	6
264.	155	116	6
288.	156	122	6
312.	—157*	126	10
336.	130	—10
360.	134
408.	—137*
Analyses at end of.....	312 Hrs.	408 Hrs.	312 Hrs.	0 Hrs.
CO ₂	33.38	33.88	1.34	0.07
O ₂	0.09	0.11	47.23	48.19
Total.....	33.47	33.99	48.58	48.26

The manometers were equilibrated at a barometric pressure of 748 mm. and temperature of 32 C.

* The organisms were granulated.

It is to be noted in the table that the O₂ had practically disappeared and was replaced by more than 33% of CO₂. Further, that pressures of —137 and —157 were attained. Recalculation of the results gave 0.692 and 0.703 as the respective real respiratory quotients of the 2 cultures. These values, however, are not correct largely because of loss of CO₂ by solution in the rubber stopper and in the medium. Exact determinations of the respiratory quotient, by means of the jar method, will be given later.

Growth in 60% O₂; Exper. 9.—The data for this experiment are given in table 9. The early manometric readings gave the impression that the germs were going to utilize this high concentration of O₂. But the readings gradually slowed

down and reached a maximum long before the O_2 was entirely consumed. It will be seen that a maximal pressure of only—88 mm. was reached, which is markedly in contrast with —137 to —157 mm. obtained with 50% O_2 in table 8. Microscopic examination showed the organisms to be granulated, or, more correctly, completely disintegrated. The development of the culture had therefore reached an end when only about 20% CO_2 was formed, and while there was still 30 or more % of O_2 present. This result finds confirmation in the following experiments and is in striking contrast with that obtained in the preceding trial. It could hardly be expected that an increment of 10% would exert so marked an effect. The fact, however, remains that 60% O_2 is definitely inhibitive.

TABLE 9
MANOMETRIC READINGS AND ANALYSES OF GROWTH OF *L. TROPICA* IN 60% OXYGEN

Tube No.	Cultures		Uninoculated	
	1	2	Final Control	Initial Control
Hrs.	Mm.	Mm.	Mm.	Mm.
0.....	0	0	0	0
24.....	0	0	0
48.....	—5	—5	0
72.....	13	13	—2
96.....	20	26	2
120.....	32	38	4
144.....	42	51	6
168.....	53	61	6
192.....	60	68	6
216.....	66	73	6
240.....	72	80	8
264.....	88	84	8
288.....	—88*	88	8
312.....	—88*	—8
Analyses at end of.....	288 Hrs.	312 Hrs.	312 Hrs.	0 Hrs.
CO_2	21.75	19.62	1.57	0.32
O_2	29.98	37.05	60.68	62.68
Total.....	61.73	56.67	62.25	62.90

The manometers were equilibrated at a barometric pressure of 740 mm. and a temperature of 31 C.

* The organisms were granulated.

Incidentally, it may be mentioned that at this concentration of oxygen Adams¹⁶ found that *B. pestis* was killed.

Growth in 80% O_2 ; Exper. 10.—The data are given in table 10. The inhibition noted in the previous experiment was now more marked. No surface growth was present, and, on final examination, though some organisms were found, they were completely disintegrated. The slight respiration observed was probably the result of protected growth in the medium, to which attention has already been called. It will suffice to point out that, in this experiment, the yield of CO_2 was only 7%, while the unused O_2 approximated 68%, and that at the same time the pressures reached only —42 mm. and —53 mm., respectively.

Growth in 100% O_2 ; Exper. 11.—The results of this test are given in table 11. No growth was observable macroscopically in the medium. Clearly, under this

TABLE 10
MANOMETRIC READINGS AND ANALYSES OF GROWTH OF *L. TROPICA* IN 80% OXYGEN

Tube No.	Cultures		Uninoculated	
			Final Control	Initial Control
	1	2	3	4
Hrs.	Mm.	Mm.	Mm.	Mm.
0.....	0	0	0	0
24.....	0	0	0
48.....	-2	-3	0
72.....	6	5	0
96.....	7	11	-10
120.....	16	17	12
144.....	19	27	12
168.....	24	30	13
192.....	27	36	13
216.....	31	41	13
240.....	35	44	14
264.....	37	48	14
288.....	39	51	14
312.....	41	53	-14
336.....	-42*	-53*
Analyses at end of.....	336 Hrs.	336 Hrs.	312 Hrs.	0 Hrs.
CO ₂	7.05	7.57	2.42	0.44
O ₂	67.86	68.31	73.41	80.23
Total.....	74.91	75.88	75.83	80.57

The manometers were equilibrated at a barometric pressure of 748 mm. and a temperature of 33 C.

* The organisms were granulated.

TABLE 11
MANOMETRIC READINGS AND ANALYSES OF GROWTH OF *L. TROPICA* IN 100% OXYGEN

Tube No.	Cultures		Uninoculated	
			Final Control	Initial Control
	1	2	3	4
Hrs.	Mm.	Mm.	Mm.	Mm.
0.....	0	0	0	0
24.....	0	0	0
48.....	0	0	0
66.....	-1	0	0
90.....	2	0	0
114.....	3	0	0
144.....	3	0	0
175.....	5	0	0
195.....	5	0	0
211.....	5*	0*	0
Analyses at end of.....	211 Hrs.	211 Hrs.	211 Hrs.	0 Hrs.
CO ₂	1.15	1.23	0.87	0.35
O ₂	97.65	96.53	97.65	98.16
Total.....	98.80	97.76	98.52	98.51

The manometers were equilibrated with a barometric pressure of 749 mm. and a temperature of 31 C.

* The organisms were granulated.

extreme concentration of O_2 , there was really no evidence of multiplication, since the manometers registered little or no change, and the analyses confirmed this fact. The small amount of CO_2 formed was undoubtedly due to cadaveric respiration.

Discussion of Results in Exper. 6-11.—The growth, and hence the respiration, of *L. tropica* was apparently stimulated by partial pressures of O_2 under 60%. But when the tension reached 60%, a distinctly injurious effect was noted, which became more and more marked as the concentration of O_2 was increased.

Just how far the volume of the gas in these tubes influenced the result, it is not possible to state, since no tests were made with anaerobic jars which have a 20-fold capacity. It is conceivable, however, that in tubes which as in this case have a capacity of about 100 c.c., the injurious effect is held back, if not done away with entirely, at certain concentrations, as, for example, 50%, by the early reduction of the relatively small volume of O_2 to a concentration that has little or no effect. Be that as it may, the limiting point appears to be reached, for the 100 c.c. volume, when the O_2 concentration is 60%.

Just how the high tensions of O_2 produce these striking results, it is not possible to state. It may be supposed that the oxidative changes within the cells are increased to a point at which the vitality of the organism becomes exhausted. This may imply inhibition or even destruction of the respiratory enzyme; or, it may mean the formation of oxidation products which are directly injurious to the cells. Certainly, the presence of CO_2 does not seem to counteract the effect due to such high O_2 tensions.

TR. LEWISI UNDER INCREASED OXYGEN TENSION

Unlike *L. tropica*, this organism rarely gives rise to a visible growth in air, and the organisms are never as numerous. And yet, when grown in air, *Tr. lewisi* consumes all of the O_2 within the culture tube almost as rapidly as does *L. tropica*. The latter, as shown by tube 7 in table 3, removed all of the O_2 within 240 hours, while the former practically accomplished the same result in 288 hours (tube 5, table 1). It might be expected from this that the 2 organisms would behave about alike under increased O_2 tension. Actually, however, *Tr. lewisi* appears to be more sensitive than *L. tropica* to an increase in the concentration of oxygen. Thus, while the latter has been shown to grow vigorously in 50% O_2 , the former is markedly inhibited in a

somewhat lower concentration. It would have been of interest to compare the behavior of cultures of *Tr. lewisi* in air with those in 25, 30, 35 and 40% O_2 , but such tests were not made.

Growth in 45% O_2 ; Exper. 12.—The details for this and the subsequent experiment were the same as those observed in the preceding tests. The inoculum as usual was a very rich one. As can be seen on reference to table 12, the negative pressure developed rather slowly, which in itself was conclusive evidence that the organisms were not growing as rapidly as under ordinary air conditions (table 1). Therefore, on analysis, it was expected that a large quantity of O_2

TABLE 12
MANOMETRIC READINGS AND ANALYSES OF GROWTH OF *TR. LEWISI* IN 45% OXYGEN

Tube No.	Cultures		Uninoculated	
			Final Control	Initial Control
	1	2	3	4
Hrs.	Mm.	Mm.	Mm.	Mm.
0.....	0	0	0	0
24.....	0	0	0
48.....	0	0	0
72.....	-1	-2	0
96.....	4	4	0
120.....	8	6	0
144.....	12	10	0
168.....	16	13	-1
192.....	20	16	2
216.....	24	18	4
240.....	26	19	6
264.....	30	21	8
288.....	32	23	11
312.....	35	24	13
336.....	38	26	11
360.....	40	26	14
384.....	-43*	-27*	-17
Analyses at end of.....	284 Hrs.	284 Hrs.	284 Hrs.	0 Hrs.
CO_2	5.45	2.96	0.85	0.11
O_2	34.85	40.80	41.77	46.23
Total.....	40.30	43.76	42.62	46.34

The manometers were equilibrated with a barometric reading of 744 mm. and a temperature of 30 C.

* The organisms were granulated.

and a small amount of CO_2 would be found, and such was actually the case. A concentration of O_2 , which is about twice that of ordinary air, is therefore clearly inhibitive.

An examination of the cultures at the end of 384 hours showed that they were granulated, but it was by no means certain that they were dead. To test this point, the cultures were aerated and then incubated for 5 days. A very rich growth developed, which indicated a mere inhibition on the part of the O_2 . As is well known, *Tr. lewisi* possesses considerable vitality, and this explains its subsequent development under the more favorable condition, that of the O_2 tension in ordinary air.

Growth in 60% O₂; Exper. 13.—The growth of the organism was again inhibited, as could be expected from the previous experiment. The culture, however, was viable at the end of 456 hours, since subsequent aeration and incubation gave a rich growth. A comparison of the manometric readings for the 2 inoculated cultures (table 13) shows an appreciable difference in the final pressures, though a similar difference is also to be noted in table 12.

These two experiments with *Tr. lewisi* are in striking agreement with those of *L. tropica*. It is evident that increased oxygen tension is distinctly inhibitive, if not injurious to these organisms. This is all the more remarkable considering the hardness of the organisms and

TABLE 13
MANOMETRIC READINGS AND ANALYSES OF GROWTH OF *TR. LEWISI* IN 60% O₂

Tube No.	Cultures		Uninoculated	
			Final Control	Initial Control
Hrs.	1	2	3	4
0.....	Mm. 0	Mm. 0	Mm. 0	Mm. 0
14.....	—3	0	0
72.....	8	—8	—1
96.....	10	9	1
144.....	15	13	3
168.....	18	14	3
192.....	20	15	4
240.....	26	18	5
288.....	30	19	6
312.....	34	—21*	6
408.....	40	7
456.....	—44*	—8
Analyses at end of.....	456 Hrs.	312 Hrs.	456 Hrs.	0 Hrs.
CO ₂	6.55	1.85	1.63	0.00
O ₂	52.32	57.73	58.95	60.52
Total.....	58.87	59.58	60.58	60.52

The manometers were equilibrated at barometric pressure of 743 mm. and the temperature 30 C.

* The organisms were granulated.

the vigor of growth which they show under ordinary air conditions. It will be seen that the former is somewhat more sensitive than the latter.

TR. LEWISI UNDER INCREASED CO₂ TENSION

It was pointed out in connection with exper. 1 that the microscopic examination made after the maximal negative pressure had been reached showed extreme granulations of the germs. At this stage the gaseous atmosphere contained about 15% of CO₂ and no O₂. Since the toxic effect of CO₂ on some protozoa has been reported by many observers,

it was desirable to establish whether the degeneration noted above was due to the increase in CO_2 or to the removal of the O_2 . With this object in view, the following experiments were made with atmospheres containing variable amounts of CO_2 and an abundant supply of O_2 .

Exper. 14.—Twelve tubes (20×150 mm.) received the usual medium, and were closed with loose cotton plugs. After inoculation, the plugs were cut off and pushed within the tubes. Two of these tubes and 5 drops of H_2O were then placed in each of 6 anaerobic jars of about 2,000 c.c. capacity.

The jars were sealed in the usual manner, but were not attached to manometers. Jars 2 to 6 were then evacuated and filled with varying concentrations

TABLE 14
EFFECTS OF VARYING CONCENTRATIONS OF CO_2 ON *Tr. lewisi*, 2 TUBES IN EACH JAR,
3 WEEKS AT 31 C.

Jar.....	1	2	3	4	5	6
Initial analyses CO_2	0.03	6.93	22.58	30.58	38.54	50.80
O_2	20.92	19.41	13.27	14.39	12.84	10.51
Final analyses CO_2	2.91	10.36	21.51	31.69	39.35	51.00
O_2	17.55	15.47	13.91*	13.30*	11.90*	9.69*
Growth.....	Rich	Rich	None	None	None	None

* The organisms showed extreme granulation.

TABLE 15
EFFECT OF VARYING CONCENTRATIONS OF CO_2 ON *L. tropica*, 2 TUBES IN EACH JAR,
3 WEEKS AT 31 C.

Jar	1	2	3	4
Initial analyses				
CO_2	0.21	9.57	20.40	29.02
O_2	20.51	19.24	16.64	14.58
Final analyses				
CO_2	2.92	13.54	23.55	30.30
O_2	17.61	14.76	13.08	11.29*
Growth.....	Rich	Rich	Rich	None

* The organisms showed extreme granulation.

of CO_2 , after which they were placed in the hot-room for 2 hours. Jar 1 contained ordinary air and served as a control. The gaseous content of each jar was then sampled and analyzed. To relieve the excess pressure which had developed within the jars because of the increased temperature, the stop-cock was momentarily opened with the tip of the cock under water. After 3 weeks' incubation at 31 C., the jars were again sampled and then opened for microscopic examination. The data are given in table 14.

On reference to table 14, it will be seen that CO_2 in a concentration of 20% or more possessed a decidedly toxic or inhibitive effect on the growth of *Tr. lewisi*. The final analyses of jars 3-6 showed practically no gas exchange, and this was confirmed by the microscopic

examination of the cultures, which revealed no evidence of multiplication. On the other hand, the tubes in jars 1 and 2 showed rich growths, and the analyses indicated an appreciable gas exchange. It is to be noted that the inhibitive effect of CO_2 was manifested in the presence of an abundance of oxygen.

L. TROPICA UNDER INCREASED CO_2 TENSION

Exper. 15.—Four jars, each containing 2 tubes which were inoculated with *L. tropica*, were used in this experiment. Jar 1 contained ordinary air and served as a control. Jars 2 to 4 were evacuated and filled with varying concentrations of CO_2 . They were then placed in the hot-room at 31 C., and, after 2 hours, the gas content of each jar was determined. As in the previous experiment, at the end of 3 weeks, the content of each jar was again analyzed. The data are given in table 15.

As could be expected from the previous experiment, CO_2 was found to be toxic for *L. tropica* even though plenty of O_2 was present. The difference in sensitivity of the 2 germs, already noted in the O_2 experiments, was again conspicuous. Thus, in jar 3, *L. tropica* produced a more marked gas exchange than did *Tr. lewisi* in the corresponding jar. It may be well to point out that in jar 2, in which the initial CO_2 tension was less than 10%, the gaseous exchange was apparently more marked than in the corresponding air controls. It would therefore appear that this amount of CO_2 possessed a stimulating action. Before accepting this interpretation, it would be necessary to know the exact air capacity of each jar.

In the case of *L. tropica*, this favoring action was also seen in jar 3, in which there was 20% of CO_2 . It is noteworthy that this concentration, which was toxic for *Tr. lewisi*, was not injurious to *L. tropica*. The CO_2 , however, began to show its unfavorable action in jar 4, in which its concentration was nearly 30%. There was no growth in this jar, whereas in the other 3 rich cultures were developed. *L. tropica* was, therefore, found to be less sensitive to increased tensions of CO_2 and of O_2 than was *Tr. lewisi*.

Tr. lewisi and L. tropica in 100% CO_2 ; Exper. 16.—This experiment was made before those given under nos. 14 and 15; and hence, before it had been learned that CO_2 , in 20 or 30% concentration sufficed to inhibit one or the other of these organisms. It follows, therefore, that no growth could be expected in this extreme concentration. The results of this experiment are presented here because of the interesting facts revealed.

It will be seen from table 16 that 8 tubes were used. These were *h*-tubes and contained the usual medium. After being attached to manometers, they were filled with 100% CO_2 , and were then placed in the hot-room at 31 C. Uninoculated tube 7 was analyzed, as an initial control, and showed over 98% CO_2 . The data are given in table 16.

The point especially to be noted is that the tubes showed a rapid rise in negative pressure, which, under ordinary air or oxygen conditions, would have indicated a very good growth. But it will be seen that control tube 8 developed the same gain in negative pressure.

The fact that the atmosphere consisted wholly or almost so of CO_2 excluded any growth other than anaerobic. But, in such case, a marked positive pressure would be expected. Here, however, there was a progressive negative rise which was not at an end even in 204 hours. Clearly, no organism could be expected to bring about such

TABLE 16
MANOMETRIC READINGS OF CULTURES OF *Tr. Lewisii* AND *L. tropica* GROWN IN 100% CO_2

Tube No.	<i>Tr. Lewisii</i>			<i>L. tropica</i>			Uninoculated	
							Initial Control	Final Control
Hrs.	Mm.	Mm.	Mm.	Mm.	Mm.	Mm.	Mm.	Mm.
0.....	0	0	0	0	0	0	0	0
2.....	-8	-12	-9	-14	-7	-12	-10
4.....	14	20	14	19	15	19	18
6.....	21	28	20	23	23	26	23
8.....	27	32	27	29	29	31	29
10.....	33	37	31	35	33	38	34
12.....	40	43	38	37	37	48	38
36.....	55	60	51	58	55	62	59
60.....	61	65	58	65	63	68	67
84.....	69	69	65	70	71	77	73
108.....	74	73	69	74	77	85	79
132.....	80	76	71	79	83	90	82
156.....	84	81	75	86	88	94	88
180.....	89	84	80	90	92	100	92
204.....	-93	-88†	-83†	-97	-101†	-107†	-98
Analyses at end of.....	204 Hrs.	204 Hrs.	0 Hrs.	204 Hrs.
CO_2	98.46	98.54	98.16	98.08
O_2	0.38	0.14	0.38	0.46
Total.....	98.46	98.68	98.54	98.54

The manometers were equilibrated at a barometric pressure of 744 mm. and temperature of 31 C.

† Not analyzed.

consumption of CO_2 . The cause for this steady loss of CO_2 had to be sought elsewhere. One factor, without doubt, was the medium which would absorb a sufficient amount until full saturation was reached. The continued rise of the negative pressure pointed, however, to some other cause.

It was shown in Part I of this series that finely cut rubber rapidly absorbed CO_2 out of an atmosphere containing 100% of this gas. Hence, the chief cause for the peculiar manometric behavior in this case was the absorption or solution of CO_2 in the rubber stopper.

It may be well to add that on subsequent aeration of the culture tubes no growth resulted; hence, the CO_2 in this concentration was toxic for the 2 organisms. It will be shown later that these organisms are not destroyed by exposure to atmospheres of N_2 or of H_2 .

The failure of the organisms to grow after aeration, in the previous experiment, suggested the possibility that some reaction product of the acid gas and the medium was the toxic agent. The dark color of the medium might indicate the formation of such a substance. To test the point, it was desirable, therefore, to submit tubes of mediums to varying concentrations of CO_2 previous to inoculation.

TABLE 17
THE MANOMETRIC READINGS AND ANALYSES OF CULTURES OF *L. TROPICA* GROWN ON MEDIUMS PREVIOUSLY EXPOSED TO PARTIAL PRESSURES OF 10, 20 AND 100% CO_2

Per cent.....	10		20		100	
Tube No.	1	2	3	4	5	6
Hrs.	Mm.	Mm.	Mm.	Mm.	Mm.	Mm.
0.....	0	0	0	0	0	0
24.....	—4	—4	—5	—6	—2	—2
48.....	20	18	17	20	18	15
67.....	36	36	33	38	31	29
91.....	50	48	47	50	37	43
115.....	51	48	50	51	37	47
143.....	—51*	—51*	—51*	—52*	—37*	—47*
Analyses at end of.....	143 Hrs.	143 Hrs.	143 Hrs.	143 Hrs.	143 Hrs.	143 Hrs.
CO_2	14.00	14.43	14.28	14.27	16.21	14.86
O_2	0.13	0.13	0.09	0.00	0.26	0.33
	14.13	14.56	14.37	14.27	16.47	15.19

The manometers were equilibrated, the barometer reading 748 mm., the temperature 31 C.

* The organisms were granulated.

L. tropica on Mediums Previously Exposed to CO_2 ; Exper. 17.—Nine *h*-tubes were prepared with the usual blood-agar medium. The uninoculated tubes were attached to manometers, which were then connected, 3 at a time, to the vacuumeter by means of the glass rake. The 3 sets were then filled with 10, 20 and 100% CO_2 , respectively, after which the tubes were disconnected, and a control from each set was taken for analysis. The remaining 6 manometers, with the attached tubes, were then placed in the hot-room and incubated for 5 days.

At the end of that period the medium in all of the tubes was dark. The manometers were then removed from the hot-room; the tubes were taken off, and, after loosening the cotton plugs, they were allowed to aerate, by diffusion, for 12 hours. Finally, to insure complete aeration, filtered air was blown over the medium and through the side-arm of each tube.

The tubes were now inoculated with *L. tropica* in the usual way. They were then reattached to the manometers and placed at 31 C. and, after 2 hours, they were equilibrated. The manometric readings and analyses are given in table 17.

The manometric readings indicated vigorous growth and multiplication, the maximal negative pressure being reached in about 91 hours, which was somewhat earlier than in exper. 2 and 3. The analyses indicated that the O_2 was practically gone, and that from 14-16% of CO_2 was present. This experiment conclusively showed that, under these conditions no injurious compound was formed, or, at least, it was not retained in the medium which had been saturated with the varying tensions of CO_2 . Further, it was evident that the destruction of the organisms, noted in the previous experiments, was due to the high concentration of CO_2 employed.

TR. LEWISI AND L. TROPICA IN THE ABSENCE OF CO_2

The previous experiments, together with those which will be presented later, show that O_2 is necessary for the growth of these organisms in the culture tube. Consequently, following the classification of Pasteur,¹⁷ they are obligative aerobes. During the period of growth carbonic acid is returned in a definite ratio to the oxygen consumed. Waste product though it is, conceivably, its presence may be necessary for the aerobic growth of these organisms. From that standpoint, it was desirable to ascertain whether or not CO_2 was essential for the development of cultures of these protozoa.

The removal of all of the CO_2 from a vigorously growing culture, as fast as it is formed, is extremely difficult. In their work with the tubercle bacillus, Moore and Williams¹⁵ placed their culture tubes under bell-jars along with soda-lime to absorb the CO_2 which was formed. While, according to their analyses, no CO_2 was present in the air of the bell-jar, it was not certain that the culture tubes, which were closed with cotton plugs, did not have some CO_2 present. Similar experiments by Adams¹⁶ showed a slow but retarded growth of the tubercle bacillus, and this fact in itself was direct evidence that CO_2 was being made. The outward diffusion of this gas from the bottom of a narrow test-tube, where it is being formed, proceeds very slowly as is to be expected from the density of the gas. Therefore, in order to remove the CO_2 as fast as it is produced, the absorbing reagent must be placed in close proximity to the source of gas production.

In view of the results presented in Part II and of the work mentioned, it was desirable to study the growth of these protozoa in the

¹⁷ Compt. rend. Acad. Sc., 1863, 56, p. 1192.

total absence of CO_2 , and, as a preliminary test of this kind, the following experiments with tube cultures were made.

Tr. lewisi in the Apparent Absence of CO_2 ; *Exper. 18*.—Nine *h*-tubes were prepared each with 5 c.c. of agar. After autoclaving, 5 c.c. of sterile 10% KOH solution were pipetted into the side-arms. The defibrinated rabbit blood was then added as usual to the agar and the mediums slanted. The tubes were then inoculated, and the cotton plugs, after having been cut off, were pushed down so as in no wise to obstruct the opening to the side-arm. The tubes were now attached to manometers and placed in the hot-room and

TABLE 18

MANOMETRIC READINGS AND ANALYSES OF CULTURES OF *TR. LEWISI* GROWN IN TUBES WITH 5 C.C. 10% KOH IN THE SIDE-ARM

Tube No.	Cultures							Uninoculated	
								Initial Control	Final Control
	1	2	3	4	5	6	7	8	9
Hrs.	Mm.	Mm.	Mm.	Mm.	Mm.	Mm.	Mm.	Mm.	Mm.
0.....	0	0	0	0	0	0	0	0	0
36.....	-7	-5	-5	-5	-5	-5	-5	-7
60.....	11	8	7	8	8	7	8	7
88.....	24	13	11	14	12	8	10	8
109.....	40	22	19	22	19	14	16	8
140.....	66	36	30	33	35	24	22	13
153.....	84	50	42	45	48	30	32	13
180.....	111	72	62	62	70	42	44	15
207.....	141	106	93	83	95	60	68	18
231.....	141	125	112	98	108	70	80	26
251.....	-141*	-146*	131	136	122	88	95	26
299.....	-143†	138	140	100	122	26
346.....	-145†	140	100	125	30
369.....	-140†	110	128	-30
465.....	-120†	-133†
Analyses at end of...	251 Hrs.	251 Hrs.	299 Hrs.	346 Hrs.	369 Hrs.	921 Hrs.	465 Hrs.	0 Hrs.	369 Hrs.
CO_2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
O_2	0.00	0.33	0.60	0.21	0.08	1.89	1.75	20.89	18.10
Total.....	0.00	0.33	0.60	0.21	0.08	1.89	1.75	20.89	18.10

The manometers were equilibrated with the barometer reading 743 mm., the temperature 31 C.

* Cultures showed considerable degeneration.

† The organisms were completely granulated.

equilibrated as usual. It will be evident from table 18 that no inhibition of growth took place, since rich cultures developed in the tubes. It is to be noted that the maximal negative pressure was nearly 3 times as great as that in *exper. 1*. The organisms consumed all or nearly all of the O_2 and produced CO_2 , which, however, was absorbed by the KOH, thus causing the high negative reading. The latter closely approximated the partial pressure of O_2 originally present in the air.

The analytical results show that no CO_2 was present, but the analyses were made after the maximal negative pressure was reached,

and hence were no index of the presence or absence of CO_2 during the period of rapid growth. This condition was taken into account in the next experiment.

L. tropica in the Apparent Absence of CO_2 ; Exper. 19.—The previous experiment was repeated using *L. tropica* as the inoculum. The manometric readings and analyses are given in table 19. The microscopic examinations which were made during the period of rapid growth (tubes 1-4) showed the organisms to be in good form and exceedingly active, notwithstanding the fact that the

TABLE 19
MANOMETRIC READINGS AND ANALYSES OF CULTURES OF *L. TROPICA* GROWN IN TUBES WITH
5 C C. 10% KOH IN THE SIDE-ARM

Tube No.	Cultures							Uninoculated	
								Initial Control	Final Control
	1	2	3	4	5	6	7	8	9
Hrs.	Mm.	Mm.	Mm.	Mm.	Mm.	Mm.	Mm.	Mm.	Mm.
0.....	0	0	0	0	0	0	0	0	0
24.....	-14	-4	-9	-4	-3	-7	-4	0
48.....	-41*	31	39	30	8	35	18	0
60.....	55	55	47	18	49	33	-5
72.....	-67*	66	57	24	62	40	7
84.....	78	70	35	75	52	8
96.....	-90†	85	41	88	65	8
110.....	97	58	99	77	10
120.....	-109*	68	105	87	9
145.....	86	116	104	12
169.....	98	122	118	13
190.....	167	127	129	15
217.....	114	129	135	17
302.....	-120†	-130†	140	20
352.....	-140†	-24
Analyses at end of...	48 Hrs.	72 Hrs.	96 Hrs.	120 Hrs.	302 Hrs.	302 Hrs.	352 Hrs.	0 Hrs.	352 Hrs.
CO_2	0.10	0.12	0.04	0.00	0.00	0.00	0.00	0.00	0.00
O_2	15.31	11.77	9.03	5.16	3.79	2.81	0.00	20.90	17.66
Total.....	15.41	11.89	9.07	5.16	3.79	2.81	0.00	20.90	17.66

The manometers were equilibrated with barometer reading 746 mm., and the temperature at 30 C.

* The cultures were rich with well-shaped organisms.

† The organisms were completely granulated.

analyses revealed little or no CO_2 . The medium in this as in the previous experiment retained its rich red color. This was the more noticeable since the ordinary cultures of *L. tropica* were characterized by marked changes in color.

In the previous experiment, no CO_2 was revealed by the analyses because the examinations were all made when the cultures had reached their full development. It was indicated in that connection that CO_2 could have been found had analyses been made during the period of

active multiplication. This experiment furnished definite proof of this fact. It will be seen on reference to table 18 that tubes 1-3 contained a small amount of CO_2 , while the subsequent cultures had none. Obviously, during the period of rapid respiration the CO_2 cannot be instantaneously absorbed; hence, a small residuum is demonstrable. It would be wrong to assume that the culture developed in the entire absence of CO_2 .

In the 2 preceding experiments, it was shown that the organisms had grown perfectly in the tubes, although alkali was present in the side-arm. It was clear that the removal of the CO_2 was not as complete or as rapid as was desired. It seemed that possibly the removal could be facilitated by employing a partial vacuum in connection with the alkali, and accordingly the following experiment was made.

L. tropica in *h*-tubes with 10% KOH in the Side-Arm, 10% O_2 Tension and a pressure of -180 mm.; Exper. 20.—Seven *h*-tubes were prepared as usual, and the medium slanted; 5 c.c. of 10% KOH were then placed in the side-arm of 6 of them, and 5 c.c. of H_2O were similarly placed in the 7th tube. Five of the tubes, including the water tube, were inoculated as usual. The other 2 tubes were kept as uninoculated controls. The tubes were then attached to the manometers which were connected by cocks 3 to a glass rake, evacuated to -350 mm., and refilled with nitrogen to zero reading, after which they were placed at 30 C. Later, they were reconnected with the vacuumeter in the hot-room and evacuated to -180 mm., thus creating in the tubes a diminished pressure, approximating 10%, which it was hoped would facilitate the removal of the CO_2 .

The data are recorded in table 20. Minute dark, round areas, indicating growth, were observed in the medium at the end of 24 hours. The appearance of the germs in tube 1, at the end of 72 hours, was excellent, but the tube again showed the presence of a small amount of CO_2 .

Clearly, the CO_2 which was produced by an actively growing culture was not instantly removed by the alkali which was present in the adjoining side-arm. The amount of this gas, however, was reduced to a small fraction of 1%. The favoring action of CO_2 , if such there was, would therefore rest with traces, rather than with any high percentage, of this gas.

The other tubes, including no. 5, the water tube, when examined 42 hours later, had a fairly rich growth, but were granulated. It will be seen from the analysis of tube 2 that no CO_2 was present. On the other hand, tube 5, which contained no alkali, had the full amount of CO_2 which could be expected considering the diminished O_2 tension originally present.

The experiment just given failed to realize the expectation as to complete and rapid removal of CO_2 . Accordingly, the method of

procedure was modified by resorting to the use of Petri dishes in the manner given in the following experiment, whereby the alkali was brought into the closest possible proximity to the culture.

L. tropica in Petri Dishes Over 5% KOH; Exper. 20 A.—In this and the following experiments a 3% agar was employed in order to have as firm a medium as possible. Each of 6 ordinary tubes received 5 c.c. of agar; after autoclaving, an equal volume of sterile, defibrinated rabbit blood was added, and the contents were then poured into sterile Petri dishes. The dishes were placed at 34 C. for 12 hours in order to set as firmly as possible. Four drops of inoculum were then placed on the blood agar in each plate, and the material was spread evenly over the surface by means of a sterile bent glass rod. Four of the plates were then inverted over 4 plates of the same diameter containing 10 c.c. of KOH. The distance between the blood agar surface and that of the alkali was less than 1 cm. Four narrow strips of adhesive tape

TABLE 20
MANOMETRIC READINGS AND ANALYSES OF CULTURES OF *L. TROPICA* IN TUBES WITH 10% O₂
TENSION, 5 C.C. 10% KOH IN SIDE-ARM AND INITIAL VACUUM OF ABOUT -180 MM.

Tube No.	Cultures				Water Control	Uninoculated	
	1	2	3	4		Initial Control	Final Control
Hrs.	Mm.	Mm.	Mm.	Mm.	Mm.	Mm.	Mm.
0.....	0	0	0	0	0	0	0
0.....	-183	-179	-189	-187	-203	-174	-176
24.....	180	184	190	190	203	176
36.....	180	193	190	201	202	176
48.....	183	195	190	209	207	176
72.....	-203*	213	211	226	215	176
96.....	222	227	228	215	176
114.....	-225*	-229†	230	-215	176
141.....	-233†	-180
Analyses at end of.....	72 Hrs.	114 Hrs.	114 Hrs.	0 Hrs.	141 Hrs.
CO ₂	0.20	0.00	6.49	0.00	0.00
O ₂	4.41	0.76	0.00	9.45	8.42
Total.....	4.61	0.76	6.49	9.45	8.42

The manometers after evacuation were placed in the hot-room, the barometer reading 729 mm., and temperature of 30 C.

* The organisms were well formed and active.

† It was impossible to withdraw a sample.

were placed vertically on the sides of the plates, thus holding them together. The remaining 2 inoculated plates were inverted, in like manner, over Petri dishes containing 10 c.c. of water. Two of the plates with alkali and one with water were placed in each of 2 anaerobic jars, which were then sealed in the usual manner. The jars were then connected to a vacuumeter and a tension of -400 mm. was produced in order to favor the liberation of the CO₂, and, at the same time, to reduce the amount of the dissolved gas. The jars were then placed at 30 C., and incubated for 6 days.

Later, on opening the jars for examination, the water plates were found to be covered with a rich continuous surface growth. The medium was dark,

but the germs were very well formed and actively motile. A striking contrast was presented by the KOH plates; each of them had but 4 colonies, from which thin streamers radiated for a distance of 1 cm. These colonies contained actively motile germs. The medium over the KOH was bright red, unlike that in the plates grown over H_2O .

This experiment, while not completely successful in demonstrating that no growth could take place in the absence of CO_2 , nevertheless appeared to be somewhat conclusive. But the factor of surface desiccation, due to the action of alkali, must not be overlooked. The great majority of organisms inoculated on the surface of the plates were clearly unable to grow under these experimental conditions. A few organisms, however, seemingly penetrated the medium, and thus securing protection, they were able to multiply although the CO_2 was completely removed from the air which was in contact with the surface of the medium. Small clumps or masses of the inoculum could exert a similar protective action.

Tr. lewisi in Petri Dishes over 5% KOH; Exper. 20 B.—The conditions for this experiment were the same as in the preceding one, except that *Tr. lewisi* was used. On opening the jars, all 6 plates were found to be covered with a continuous luxuriant growth of active motile germs. The medium over the water was dark, while that over the KOH was bright red.

The results of this experiment gave no support to the view that CO_2 was necessary for growth, since perfect cultures were obtained in the apparent absence of CO_2 . With the expectation of securing a more definite outcome, the method of procedure was modified, for the next 2 experiments, by substituting a 10% KOH solution for that previously used.

L. tropica in Petri Dishes Over 10% KOH; Exper. 20 C.—Six tubes were prepared as in exper. 20 A, the total volume of medium in each tube, however, being 15 c.c., to lessen the desiccation arising by the use of a more concentrated reagent. The medium was poured into Petri dishes, which were allowed to stand 12 hours at 34 C. The inoculated plates were then inverted over like dishes containing 10% KOH, and 2 water controls were made as before. The plates were now placed in anaerobic jars, which were evacuated to -400 mm. They were then incubated at 30 C. for 6 days. When examined, the water controls were found to be covered with a luxuriant growth of active germs. The KOH plates, on the other hand, were dotted with small round isolated colonies, about 30 per plate. Examination of these colonies showed perfect organisms. Seemingly little desiccation of the medium was observed, but the plates were not weighed at the start and again at the end of the experi-

ment to determine the exact loss. The results in this experiment are confirmatory of those obtained in exper. 20 A, in which the same organism was used.

Tr. lewisi in Petri Dishes Over 10% KOH; Exper. 20 D.—Six plates of medium were prepared as in the previous experiment, *Tr. lewisi* being used as the inoculum. The plates were placed over 10% KOH and H₂O as before. They were then placed in anaerobic jars, evacuated to -400 mm. and incubated 8 days. On examination, the KOH plates showed no indications of growth, while the water plates were covered with a rich surface growth. This experiment was apparently successful in demonstrating that no growth could take place when the CO₂ was completely removed. Unfortunately, the extent of desiccation was not determined.

In the hope of obtaining more consistent results than those given by the preceding experiments, it was decided to make similar tests substituting serum agar for the medium which had been used. The results of these tests are given in the next 2 experiments. It will be seen that, as regards the need of CO₂, the outcome was even less satisfactory than in any of the preceding tests.

L. tropica on Serum Agar, Over 10% KOH; Exper. 20 E.—To each of 3 tubes containing 10 c.c. of agar, 5 c.c. of rabbit blood serum were added, and the mixture poured into Petri dishes. After standing for 12 hours at 34 C., the plates were inoculated with 2 drops of a culture, and the inoculum was spread over the surface as before. Two of the plates were then inverted over like dishes containing 10 c.c. of 10% KOH. The other plate was placed over water. The plates were now placed in an anaerobic jar and incubated for 6 days. On examination, a rich growth was present on all 3 plates.

Tr. lewisi on Serum Agar, Over 10% KOH; Exper. 20 F.—This experiment was prepared in the same way as the previous one, using *Tr. lewisi* as the inoculum. Subsequent examination at the end of 6 days likewise showed rich surface growths on all the plates.

The result of the last 2 experiments in which serum was used instead of whole blood was difficult to explain. The amount of serum was somewhat higher than that present in the 7.5 c.c. of blood used in the preceding tests. It is possible that this represents a higher concentration of carbonates and phosphates, which may have some bearing on the results. On the other hand, it is more likely that the serum agar, by reason of its greater softness, more readily withstood the desiccating action of the alkali, and thus promoted the growth of the organisms.

The 6 foregoing plate experiments may briefly be summarized by stating that in 3 trials with *Tr. lewisi*, one resulted in no growth. In the corresponding 3 tests with *L. tropica*, 2 showed marked inhibition. On the other hand, the 3 trials with *h*-tubes (exper. 18-20) gave

growths although alkali was present in the side-arms. In these, however, minimal amounts of CO_2 were present during the active stage of multiplication because the absorption of this gas was relatively slower than its production.

In the plate experiments, the conditions for the rapid absorption of CO_2 were good, but the results were somewhat masked by the desiccation of the medium. It must be evident that an organism cannot possibly grow on a dry surface. The immediate effect of the presence

TABLE 21
MANOMETRIC READINGS OF CULTURES OF *Tr. Lewisii* AND *L. tropica* GROWN IN 100% H_2

Tube No.	<i>Tr. Lewisii</i>			<i>L. tropica</i>			Uninoculated	
	1	2	3	4	5	6	Initial Control	Final Control
Hrs.	Mm.	Mm.	Mm.	Mm.	Mm.	Mm.	Mm.	Mm.
0.....	0	0	0	0	0	0	0	0
24.....	+1	0	+1	0	+2	0	0
48.....	+1	0	0	-1	+2	0	+1
72.....	+2	0	0	0	0	-1	+1
96.....	+1	-1	0	+1	0	-1	-1
120.....	+1	-1	0	0	0	0	-1
144.....	0	-1	-1	0	0	-1	-1
168.....	0	-1	-1	0	+1	-1	-1
192.....	0	-2	-1	-1	0	-1	-2
216.....	-1	-2	0	-1	0	-1	-2
240.....	-1	-2	+1	-1	+1	-1	-2
264.....	-2	-3	0	-2	-1	-2	-3
288.....	-2	-3	-1	-2	-1	-2	-3
336.....	-2†	-2	-1†	-2†	-3	-2†	-3
Analyses at end of.....	336 Hrs.	336 Hrs.	0 Hrs.	336 Hrs.
CO_2	0.16	0.19	0.05	0.15
O_2	0.03	0.03	0.00	0.00
Total.....	0.19	0.22	0.05	0.15

The manometers were equilibrated at a barometric pressure of 742 mm. and a temperature of 30 C.

† Not analyzed.

of alkali is to remove all of the moisture on the surface of the medium, and hence inhibition of growth must necessarily take place. It should be added that results similar to those in these experiments were obtained with *B. tuberculosis* (Part II).

TR. LEWISII AND L. TROPICA IN THE ABSENCE OF O_2

In all of the experiments which have hitherto been made, these organisms were grown under aerobic conditions. No actual tests had been made to determine whether they were capable of growing under anaerobic conditions. Accordingly, the following experiments with

H₂, N₂ and CO₂ were planned to determine whether they could be grown in the total absence of O₂. The results obtained with CO₂ have already been presented under exper. 16.

Tr. lewisi and *L. tropica* in 100% H₂; *Exper. 21.*—For this experiment, *h*-tubes containing blood agar were used. Of these, 3 were inoculated with *Tr. lewisi* and 3 with *L. tropica*, and 2 served as uninoculated controls. These were then attached to manometers and filled with H₂, then placed in the hot-room and equilibrated as usual.

The results of this experiment are given in table 21. It will be seen that the manometric readings were practically nil. Any growth under these

TABLE 22
MANOMETRIC READINGS OF CULTURES OF *TR. LEWISI* AND *L. TROPICA* GROWN IN 100% N₂

Tube No.	Tr. Lewisii			L. tropica			Uninoculated	
							Initial Control	Final Control
	1	2	3	4	5	6	7	8
Hrs.	Mm.	Mm.	Mm.	Mm.	Mm.	Mm.	Mm.	Mm.
0.....	0	0	0	0	0	0	0	0
12.....	0	+1	0	+1	0	0	0
36.....	0	+2	0	+1	0	0	0
60.....	0	+1	0	0	-1	-1	0
84.....	+1	0	+1	0	-1	-1	-1
108.....	+2	0	+1	0	-1	0	0
132.....	+2	0	0	+1	-1	-1	0
156.....	+3	0	+1	0	0	+1	-2
180.....	+3	+1	0	0	-1	0	-3
204.....	+2	0	-1	-1	-1	0	-3
228.....	+1	-1	-2	-2	-1	-1	-3
252.....	+1	-1	-2	-1	-1	-1	-3
276.....	0†	-1†	-2	-1†	-2	-2†	-3
Analyses at end of.....	324 Hrs.	324 Hrs.	0 Hrs.	324 Hrs.
CO ₂	0.18	0.16	0.01	0.11
O ₂	0.00	0.00	0.17	0.14
Total.....	0.18	0.16	0.18	0.25

The manometers were equilibrated at a barometric pressure of 740 mm. and a temperature of 30 C.
† Not analyzed.

conditions would be indicated by a positive reading, which should increase with the duration of the test. The experiment extended over 14 days. No growth was visible macroscopically, and the manometric readings confirmed its absence. The analyses of 2 of the inoculated tubes showed essentially the same composition as that of the 2 controls.

At the close of the experiment the tubes were disconnected and examined; they showed few motile forms. They were then set aside in the incubator, and gave a very rich growth, showing that H₂ had no toxic effect but that the organisms were merely inhibited by the lack of O₂.

Tr. lewisi and *L. tropica* in 100% N₂; *Exper. 22.*—This experiment was the same as the preceding one except for the substitution of N₂. As seen in table 22, the results were the same, the manometers indicating no growth.

On disconnecting, a few motile forms were found in each of the tubes. These, on further incubation in air, developed very rich growths, showing, as in the experiment with H_2 , that N_2 itself was not toxic. The absence of growth was therefore wholly due to the lack of O_2 .

TR. LEWISI AND L. TROPICA ON SERUM AGAR

Tr. brucei was first cultivated by Novy and MacNeal in 1904. The medium was the same as that used in this work. In 50 attempts, Novy and MacNeal obtained only 4 positive results. Smedley¹⁸ found that 3 out of 10 attempts were positive. Behrens,⁹ because of the inconsistent results, attempted to improve the medium. His best results were obtained by the employment of a dialyzed meat extract, made as follows:

One hundred and twenty-five gm. chopped beef, and 250 c.c. of water were allowed to digest over night in the cold. The mixture was then strained and the extract was boiled and filtered. The filtrate was then dialyzed for 24 hours in a large collodium sac against running distilled water. The dialyzed contents of the sac were then diluted to 1 liter with distilled water, and 2% peptone, 0.5% NaCl, 0.01% $CaCl_2$, 10 c.c. of a normal solution of Na_2CO_3 , and 2% agar were added. To this nutrient agar, rabbit blood serum was added in a ratio of 2 to 1. On inoculation, he found that this serum agar gave 100% of successful cultures. This medium was used in attempts to cultivate the trypanosomes of sleeping sickness, caderas, surra and dourine, but unsuccessfully.

It was desirable to ascertain the effect, if any, on the gas exchange, of the substitution of serum for whole blood. With this object in view, experiments were made in which serum agar was used in place of blood agar, as had hitherto been the case.

To obtain the serum, the rabbit was bled from the carotid artery into the sterile pipet. Instead of defibrinating, as usual, the pipets were now inclined so as to give, on coagulation, a maximal surface. After the blood had clotted around the defibrinating rod, the pipets were set aside in a vertical position for 24 hours at 10 C. The clear serum, thus obtained, was drawn up into sterile Pasteur bulb pipets, and added to the melted agar in *h*-tubes in the proportion of 1:1, the agar having been previously cooled to 50 C.

Exper. 23.—The *h*-tubes, prepared as indicated above, were slanted for 12 hours. They were inoculated with the 2 organisms, attached to manometers, placed in the hot-room, and then finally equilibrated.

The results of this experiment are given in table 23. As regards the manometric readings, it will be seen that the 2 organisms developed the same maximal negative pressure of -45 mm. In the case of *Tr. lewisi*, this point was reached in about twice the time as that with *L. tropica*, which was in accord with the well-known fact that the former multiplied more slowly. The manometric reading of the final control, tube 5, remained zero throughout

¹⁸ Jour. Hyg., 1905, 5, p. 38.

TABLE 23
MANOMETRIC READINGS AND ANALYSES OF CULTURES OF TR. LEWISI AND L. TROPICA GROWN ON RABBIT SERUM AGAR

Tube No.	L. tropica		Tr. Lewisi		Uninoculated	
	1	2	3	4	Final Control	Initial Control
Hrs.	Mm.	Mm.	Mm.	Mm.	Mm.	Mm.
0.....	0	0	0	0	0	0
7.....	0	0	0	0	0	—
19.....	—5	—6	0	0	0
24.....	10	12	0	0	0
43.....	29	31	—2	—2	0
48.....	30	35	2	2	0
67.....	41	45	6	6	0
82.....	43	46	10	13	0
96.....	—45*	—46*	14	19	0
114.....	23	30	0
138.....	30	40	0
162.....	36	44	0
186.....	42	45	0
210.....	—45*	—45*	0
Analyses at end of....	96 Hrs.	96 Hrs.	210 Hrs.	210 Hrs.	210 Hrs.	0 Hrs.
CO ₂	14.56	14.69	14.46	14.56	0.57	0.20
O ₂	0.03	0.18	0.20	0.23	20.08	20.78
Total.....	14.59	14.87	14.66	14.79	20.65	20.98

The manometers were equilibrated at a barometric reading of 747 mm., a temperature of 31 C.

* The cultures were very rich with motile well-formed germs.

TABLE 23a
CALCULATED RESPIRATORY QUOTIENTS AND MANOMETRIC READINGS FOR EXPER. 23

Tube No.	L. tropica		Tr. Lewisi	
	1	2	3	4
Analyses CO ₂	14.56	14.69	14.46	14.56
O ₂	0.03	0.18	0.20	0.23
N ₂	14.59	14.87	14.66	14.79
Total.....	85.41	85.13	85.34	85.21
Corrected analyses CO ₂	13.47	13.60	13.32	13.50
O ₂	0.03	0.16	0.18	0.21
N ₂	13.50	13.76	13.50	13.71
Total.....	79.02	79.02	79.02	79.02
Real gain CO ₂	92.52	92.78	92.52	92.73
Real loss O ₂	13.27	13.40	13.12	13.30
Respiratory quotient.....	20.75	20.62	20.60	20.57
Calc. man. reading.....	0.639	0.649	0.637	0.645
Corr. obs. man. readings.....	—53.4	—51.4	—53.4	—51.9
	—45.9	—46.87	—46.26	—46.6

Corrected analyses = analytical value × nitrogen factor.
Calculated manometric readings = real loss × (B — aqueous tension).

TABLE 24

MANOMETRIC READINGS AND ANALYSES OF CULTURES OF *Tr. Lewisii* AND *L. tropica* GROWN ON SERUM DIALYZED AGAR

Tube No.	<i>L. tropica</i>		<i>Tr. Lewisii</i>		Uninoculated	
	1	2	3	4	Final Control	Initial Control
Hrs.	Mm.	Mm.	Mm.	Mm.	Mm.	Mm.
0.....	0	0	0	0	0	0
60.....	-12	-2	-2	0	0
72.....	16	4	4	4	0
92.....	24	11	8	6	0
116.....	30	18	16	18	0
140.....	34	25	24	27	0
164.....	38	31	32	36	0
176.....	39	33	36	40	0
188.....	41	37	38	-45†	0
212.....	-41*	40	-45†	0
224.....	45	0
260.....	-45*	0
Analyses at end of.....	212 Hrs.	260 Hrs.	212 Hrs.	188 Hrs.	260 Hrs.	0 Hrs.
CO ₂	15.09	13.46	14.73	14.41	0.72	0.19
O ₂	0.41	1.12	0.31	0.38	20.26	20.74
Total.....	15.50	14.58	15.04	14.79	20.98	20.93

The manometers were equilibrated at a barometric reading of 750 mm., temperature 31 C.

* The organisms were completely granulated.

† The organisms were well formed and active.

TABLE 24a

CALCULATED RESPIRATORY QUOTIENTS AND MANOMETRIC READINGS FOR EXPER. 24

Tube No.	<i>L. tropica</i>		<i>Tr. Lewisii</i>	
	1	2	3	4
Analyses CO ₂	15.09	13.46	14.73	14.41
O ₂	0.41	1.12	0.31	0.38
N ₂	15.50	14.58	15.04	14.79
Total.....	84.50	85.42	84.96	85.21
Corrected analyses CO ₂	14.11	12.46	13.70	13.37
O ₂	0.38	1.03	0.28	0.35
N ₂	14.49	13.49	13.98	13.72
Total.....	79.07	79.07	79.07	79.07
Real gain CO ₂	93.56	92.56	93.05	92.79
Real loss O ₂	13.92	12.27	13.51	13.18
Respiratory quotient.....	20.36	19.71	20.46	20.39
Calc. man. readings.....	0.683	0.623	0.660	0.645
Corr. obs. man. readings.....	-46.12	-53.3	-49.78	-51.6
	-41.54	-46.03	-45.76	-45.72

Corrected analyses = analytical value × nitrogen factor.

Calculated manometric readings = real loss × (B - aqueous tension).

the experiment. On reference to tables 1 and 3, in which blood agar was used, it will be seen that the corresponding controls gradually developed a slight negative pressure finally reaching -8 mm. This would indicate that the whole blood, because of its cellular elements or because of the method of defibrination, may carry on some respiratory changes which are precluded in the serum agar.

The analyses given in the table are particularly interesting. They revealed the fact that practically all of the O_2 was gone, and that about 14.5% of CO_2 was present. The rate of respiration in this experiment was faster than in tables 1 and 3, but whether this was due to a softer medium or to a larger inoculum, it is not possible to say.

The analytical results obtained in this experiment were recalculated to the nitrogen basis for the purpose of obtaining the real respiratory quotient. It will be seen on reference to table 23a that the quotient for *L. tropica* was found to be 0.639 and 0.649, while that for *Tr. lewisi* gave similar values, namely, 0.637 and 0.645. It should be pointed out, however, that this real quotient is uncorrected for the CO_2 which was dissolved in the medium. More exact determinations of the quotients will be given later.

Microscopically, the germs were actively motile and well shaped. It follows that serum agar was in every respect as good a culture medium as, if not better than, blood agar. No difference, however, was observable in the gas exchange.

Exper. 24.—In the preceding experiment, the serum was added to agar which was prepared in the usual way. As pointed out, however, Behrens⁹ obtained unusually good results in the cultivation of *Tr. brucei* by employing a dialyzed meat extract. To determine whether this had any advantage over the ordinary agar medium the following experiment was made in which his technic was fully observed. In all other respects, this experiment duplicated the previous one.

The results were practically the same, and from table 24 and 24a it may be concluded that this medium has no advantage over the preceding. The gas exchange was in no wise different, as indicated by the manometric readings of -41 to -45 mm.

The average of the real respiratory quotients of each organism was slightly higher than the corresponding values given in table 23a. Thus, the average value for *L. tropica* was 0.653, as against 0.644 in the former experiment; that for *Tr. lewisi* was 0.652 as contrasted with 0.642.

TR. LEWISI AND L. TROPICA ON GLUCOSE MEDIUMS

A considerable literature exists relative to the sparing action of utilizable carbohydrate as regards the proteins in cultures of bacteria. It may be sufficient to mention that Kendall¹⁹ and his co-workers have

¹⁹ *Physiol. Rev.*, 1923, 3, p. 438.

made extensive determinations of the amount of ammonia which is produced in various cultures in the presence or absence of carbohydrate.

In all of the previous experiments in which defibrinated blood or serum was used there was obviously a small amount of carbohydrate present, but probably not to an extent to materially influence the gas exchange. It could be expected that the addition of glucose to such mediums would produce an appreciable difference in the ratio of CO_2 made and O_2 consumed; in short, in the respiratory quotient.

The experiments which are to be described were intended to bring out the effect of the addition of glucose to the mediums.

The ordinary glucose mediums are not favorable for the growth of these organisms. They will survive for a few days when planted on plain glucose agar, or in glucose broth, but very little multiplication, if any, takes place. In the experiments which follow, 4% of glucose was added to the agar, and the reaction was readjusted to P_H 7.4. After the addition of defibrinated rabbit blood, or serum, the final concentration of glucose became 2%.

Tr. lewisi and *L. tropica* on 2% glucose blood agar; *Exper. 25*:—Five *h*-tubes were prepared with this medium. Two were inoculated with *Tr. lewisi* and 2 with *L. tropica*, while the 5th tube was used for an initial control. The tubes were then attached to manometers, placed in the hot-room, and, after 2 hours, equilibrated.

The manometric readings and analyses are given in table 25. Indications of growth were observed at the end of 29 hours by the appearance of dark areas, which rapidly increased in size. At the same time, colonies or masses of organisms became recognizable.

On examination of table 25, a striking fact will be noted, namely, that the maximal negative pressures were only about one-half of those observed in experiments with the usual mediums. The explanation of this behavior was supplied by the analyses. These showed the O_2 to be practically exhausted and replaced by about 18% of CO_2 . This high percentage meant that the real loss was less than in previous experiments, and hence the observed manometric readings were necessarily lower.

A recalculation of the analytical values to the nitrogen basis brings out clearly the effect due to the presence of glucose. It will be seen from table 25*a*, for example, that the real gain in CO_2 was 16.5-17.5%, whereas that in table 24 was less than 13.5. This increase in CO_2 at once was indicative of a higher respiratory quotient and, as seen from the table, the real quotients were 0.8 or higher, whereas in table 24*a* they were 0.6 or slightly more. Here, as in previous experiments, the respiratory quotients were lower than they really should be, for the reason that more or less CO_2 was dissolved by the medium. Had

TABLE 25
MANOMETRIC READINGS AND ANALYSES OF CULTURES OF TR. LEWISI AND L. TROPICA GROWN ON 2% GLUCOSE BLOOD AGAR

Tube No.	Tr. Lewisi		L. tropica		Uninoculated
					Initial Control
	1	2	3	4	5
Hrs.	Mm.	Mm.	Mm.	Mm.	Mm.
0.....	0	0	0	0	0
29.....	-5	0	0*	0*
63.....	2	0	-2	0
75.....	3	0	3	0
87.....	4	0	7	0
99.....	7	0	9	-3
111.....	13	-4	15	7
123.....	13	6	18	9
135.....	18	10	21	11
147.....	18	12	23†	13†
167.....	20	17	25	-18‡
183.....	-20‡	-18‡	-25‡
Analyses at end of.....	183 Hrs.	183 Hrs.	183 Hrs.	167 Hrs.	0 Hrs.
CO ₂	18.36	18.35	17.56	17.05	0.34
O ₂	0.13	0.15	0.05	1.56	20.43
Total.....	18.49	18.50	17.61	18.61	20.77

The manometers were equilibrated at a barometric reading of 753 mm., a temperature of 31 C.
* Large dark areas on surface of mediums.
† The surface of the mediums was covered with a heavy mucus-like white growth.
‡ Microscopic examination showed the presence of well-shaped partially granulated active germs.

TABLE 25a
CALCULATED RESPIRATORY QUOTIENTS AND MANOMETRIC READINGS FOR EXPER. 25

Tube No.	Tr. Lewisi		L. tropica	
	1	2	3	4
Analyses CO ₂	18.36	18.35	17.56	17.05
O ₂	0.13	0.15	0.05	1.56
N ₂	18.49	18.56	17.61	18.61
	81.51	81.44	82.39	81.39
Total.....	100.00	100.00	100.00	100.00
Corrected analyses CO ₂	17.84	17.84	16.88	16.59
O ₂	0.12	0.14	0.04	1.51
N ₂	17.96	17.98	16.92	18.10
	79.23	79.23	79.23	79.23
Total.....	97.19	97.21	96.15	97.33
Real gain CO ₂	17.50	17.50	16.54	16.25
Real loss O ₂	20.31	20.29	20.39	18.92
Respiratory quotient.....	0.861	0.862	0.811	0.858
Calc. man. reading.....	-20.2	-20.1	-27.7	-20.0
Corr. obs. man. reading.....	-20.2	-18.5	-25.27	-18.49

Corrected analyses = analytical value × nitrogen factor.
Calculated manometric readings = real loss × (B - aqueous tension).

TABLE 26
MANOMETRIC READINGS AND ANALYSES OF CULTURES OF *L. TROPICA* GROWN ON
2% GLUCOSE SERUM AGAR

Tube No.	Cultures		Control
	1	2	3
Hrs.	Mm.	Mm.	Mm.
0.....	0	0	0
22.....	-2	0
34.....	4	-2
46.....	6	2
58.....	10	6
70.....	13	8
82.....	15	11
94.....	17	12
106.....	20	16
118.....	23	19
130.....	25	13
142.....	27	25
154.....	28	27
166.....	30	30
178.....	30	32
190.....	32	35
202.....	30	35
214.....	-32*	-38*
Analyses at end of.....	214 Hrs.	214 Hrs.	0 Hrs.
CO ₂	17.64	15.25	0.32
O ₂	0.13	1.24	20.69
Total.....	17.77	16.50	21.01

The manometers were equilibrated, the barometer reading 747 mm., temperature 31 C.

* The organisms were completely granulated.

TABLE 26a
CALCULATED RESPIRATORY QUOTIENTS AND MANOMETRIC READINGS FOR EXPER. 26

Tube No.	1	2
Analyses CO ₂	17.64	15.26
O ₂	0.13	1.24
N ₂	82.23	83.50
Total.....	100.00	100.00
Corrected analyses CO ₂	16.94	14.43
O ₂	0.12	1.17
N ₂	17.06	15.60
Total.....	78.99	78.99
Real gain CO ₂	16.62	14.11
Real loss O ₂	20.57	19.52
Respiratory quotient.....	0.807	0.723
Calculated man. reading.....	-28.1	-38.5
Corrected observed manometric reading.....	-32.54	-38.6

Corrected analyses = analytical value × nitrogen factor.

Calculated manometric reading = real loss × (B — aqueous tension).

TABLE 27
MANOMETRIC READINGS AND ANALYSES OF CULTURES OF TR. LEWISI GROWN ON 2% GLUCOSE
SERUM AGAR AND ON SERUM AGAR

	Glucose Serum Agar		Plain Serum Agar		Uninoculated	
	1	2	3	4	Initial Control	Final Control
Tube No.	1	2	3	4	5	6
Hrs.	Mm.	Mm.	Mm.	Mm.	Mm.	Mm.
0.....	0	0	0	0	0	0
65.....	0	-2	-1	-2	0
89.....	-7	9	6	5	0
102.....	7	11	7	6	0
125.....	14	16	15	13	0
137.....	16	19	20	16	0
149.....	19	25	23	22	0
173.....	20	30	31	31	0
198.....	28	38	33	40	-2
209.....	30	39	36	44	2
258.....	25	35	-43*	52	3
282.....	-25*	-35*	-52*	-3
Analyses at end of.....	282 Hrs.	282 Hrs.	258 Hrs.	282 Hrs.	0 Hrs.	282 Hrs.
CO ₂	17.00	16.80	14.41	13.72	0.08	0.70
O ₂	0.23	0.00	0.18	0.06	20.01	19.12
Total.....	17.23	16.80	14.59	13.78	20.09	19.82

The manometers were equilibrated at a barometric reading of 749 mm., temperature 30 C.
* The organisms were granulated.

TABLE 27a
CALCULATED RESPIRATORY QUOTIENTS AND MANOMETRIC READINGS FOR EXPER. 27

	Glucose Serum Agar		Plain Serum Agar	
	1	2	3	4
Tube No.	1	2	3	4
Analyses CO ₂	17.00	16.80	14.41	13.72
O ₂	0.23	0.00	0.18	0.06
N ₂	17.23	16.80	14.59	13.78
Total.....	82.77	83.20	85.41	86.22
Corrected analyses CO ₂	16.41	16.13	13.48	12.71
O ₂	0.22	0.00	0.16	0.05
N ₂	16.63	16.13	13.64	12.76
Total.....	79.91	79.91	79.91	79.91
Real gain CO ₂	16.33	16.04	13.40	12.62
Real loss O ₂	19.79	20.01	19.85	19.96
Respiratory quotient.....	0.825	0.798	0.675	0.633
Calc. man. reading.....	-24.74	-28.3	-45.4	-51.71
Corr. obs. man. reading.....	-25.39	-35.46	-44.1	-52.75

Corrected analyses = analytical value × nitrogen factor.
Calculated manometric readings = real loss × (B - aqueous tension).

the dissolved CO_2 been determined in these tests, the corrected real respiratory quotients, in all probability, would have been found to approximate 1, which is the theoretical value for glucose (tables 30, 31 and 33).

L. tropica on 2% Glucose Serum Agar. *Exper. 26.*—In view of the interesting results observed in the previous experiment, it was repeated, but with the substitution of serum for the blood. The data are given in tables 26 and 26a.

It will be seen that the manometric readings were somewhat higher than with blood glucose medium but less than those with plain serum agar. The analyses showed a high CO_2 content, but the respiratory quotients, though high, did not agree as well as before.

Tr. lewisi on 2% Glucose Serum Agar. *Exper. 27.*—This experiment was a continuation of the preceding one except that *Tr. lewisi* was used, and an additional feature was added in that the growth on glucose serum agar was compared with that on plain serum agar, under otherwise identical conditions.

Six tubes were prepared, 4 of which had glucose serum agar and 2 had plain serum agar. Two of the glucose tubes were reserved for controls, and the remaining tubes were inoculated with rich material.

The *h*-tubes were then attached to manometers, placed in the hot-room, and, after the usual period of 2 hours, were equilibrated.

The tubes when examined at the close of the experiment showed a very rich growth, though somewhat granulated. It will be seen from table 27 that the manometric readings of the glucose agar tubes, because of the relatively larger amount of CO_2 , were appreciably lower than those which were attached to tubes of plain serum agar. It will further be noted that the CO_2 content in the former was about 3% higher than in the latter, indicating again that the respiratory quotient of the growth on glucose medium would be higher than that on the plain medium.

Reference to table 27a will reveal that the real quotients were about 0.8 for the former and about 0.65 for the latter, which values agreed closely with those given in table 23a. As pointed out heretofore, the real quotients thus obtained are not final, since, in the calculation, only the gaseous CO_2 is taken into consideration. The true or corrected real quotients are obtained by including all of CO_2 which is taken up by the rubber and by the medium.

CORRECTED REAL RESPIRATORY QUOTIENTS

The fact that man and animals consume oxygen and return CO_2 was shown by Lavoisier³ in 1777. He proved animal heat to be due in a large part to the combustion of carbon to CO_2 in the animal body, and subsequently promulgated the combustion theory of respiration. Dulong,²⁰ continuing the work, found a difference in the volume of CO_2 returned per volume of O_2 consumed in dogs, rabbits and fowls. He suggested that this might be due to a difference in the character of the food. Dulong's suggestion was substantiated by Regnault and Reiset.¹³

readily seen in the experiments which follow. For a full discussion

²⁰ Ann. d. Chim. et d. Phys., 1841, 3, p. 1.

On the assumption that the oxidation is completed to CO_2 and H_2O , the theoretical respiratory quotient of a carbohydrate is 1.0; of a fat, 0.71 and of a protein, 0.8. If the respiratory quotient is high, carbohydrate is supposedly being oxidized in the body, while if low, fat is undergoing the change. It is clear that in anaerobic respiration CO_2 is liberated, although no atmospheric O_2 is consumed.

Moore²¹ and his men, working on the respiratory quotients of marine animals, found in all cases a quotient greater than 1. Their observations were based on the determination of dissolved O_2 in seawater and of the CO_2 produced. While they accounted for the high quotients by assuming a conversion of carbohydrate to fat with the liberation of CO_2 , the consumption of O_2 in the reaction being smaller in amount, it is not clear that they excluded the action of anaerobic micro-organisms which, it is reasonable to believe, were present. In work of this kind, it may be difficult to evaluate correctly the results obtained unless the growth of micro-organisms is excluded.

The calculation of the true real respiratory quotients necessitates exact determination of all of the CO_2 produced and of the O_2 consumed. Krogh,²² in his study of exact respiration in man, pointed out the marked effect of such a factor as vapor tension. Later, Carpenter²³ compared the recent respiratory methods and showed that the great variance in results was due to this and other factors mentioned by Krogh. The respiratory quotients of micro-organisms, as a rule, have been computed from the analysis of the gases over the cultures, but the values thus obtained can at best be considered only approximate. The direct use of the uncorrected analytical data is wrong, since it gives, as pointed out in Part I, the apparent respiratory quotient, which is higher than the real quotient. Another common error is the failure to determine the amount of CO_2 dissolved in the medium. When the amount of CO_2 which is held in solution and in combination in the medium is taken into consideration, it will be found that the corrected real respiratory quotient is greater than the real, and may even exceed the apparent quotient.

Another point which must be considered is the period of incubation, which with some organisms may be without appreciable effect. With others, on account of secondary changes in the medium, such as decarboxylation, a longer duration results in an increased CO_2 production and, hence, in a higher final value. This fact will be

²¹ Bio-chem. Jour., 1912, 6, p. 255.

²² Bio-Chem. Ztschr., 1907, 7, p. 24.

²³ Carnegie Institution, Washington; Publication No. 216.

readily seen in the experiments which follow. For a full discussion of the methods and principles involved in the determination of the true quotient, reference is made to Part I.

In the series of experiments which are to be given, the procedure was essentially the same, the main difference being in the period of incubation and in the culture used.

Corrected Real Respiratory Quotient of Tr. lewisi on Blood Agar.—Four tubes (20 x 150 mm.) of blood agar were prepared as usual but with very loose cotton plugs. After having been slanted 12 hours, 2 of the tubes were inoculated, each with 2 drops of a culture of *Tr. lewisi*, and the inoculum was spread with a sterile platinum wire. The 2 uninoculated tubes were used for the control determination of CO₂ in the medium. The 2 inoculated tubes were placed in an anaerobic jar and, at the same time, 5 drops of distilled water at 80 C. were put on the bottom to hasten the production of aqueous tension. Thereupon the jar was closed, clamped, and attached to a manometer by a rubber stopper (fig. 6, Part I). The manometer and jar were mounted on a common support to relieve all strain from the connection. The apparatus was then placed in the hot-room, with stop-cocks 1 and 3 closed. A positive pressure developed, which was partially relieved by the withdrawal of samples for analysis. After sampling, the manometer was equilibrated. Readings were taken from time to time, and at the end of 28 days the gas content was analyzed. When the analysis was completed, the jar was opened, and the CO₂ in the culture tubes was determined by the aeration method described in Part I. The analytical data and calculations are given under exper. 4 and in table 28.

It should be noted that the corrected real respiratory quotient is based on gas volumes under standard conditions of temperature and pressure. Assuming protein to be the source of energy, it will be seen that the quotient 0.888 is higher than would be expected. This suggested the possibility that some CO₂ was made by some other process than respiration. Before discussing the reason for this high value, a similar experiment with *L. tropica* will be given.

Corrected Real Respiratory Quotient of L. tropica on Blood Agar.—This experiment was carried on at the same time as the previous one. The data are given in table 29 under exper. 4. The corrected real respiratory quotient (0.907) was even higher than that just given for *Tr. lewisi*. As suggested in connection with that experiment, it is not reasonable to believe that this value actually represents the respiratory process.

The question arose as to what could be the source of CO₂ other than respiration. If enzymes, whether made by the germs or present in the blood, were acting on the medium, the amount of CO₂ derived from this source probably should be less with a shorter period of incubation. To test out this point, 6 additional experiments were made, 3 with *Tr. lewisi* and 3 with *L. tropica*. In these, the periods of incubation were varied from 7 to 21 days. To avoid repetition, it may be well to state that in each of these tests the procedure was the same as that given under exper. 28. The data and the results obtained in these 8 experiments, in which the culture medium was blood agar, are given in tables 28 and 29.

Results with Tr. lewisi.—The 4 experiments given in table 28 extended over 7, 11, 14 and 28 days, respectively. It will be noted

that manometric readings increased slowly with the duration of the test. Since active multiplication must have ceased after about 10 days, it follows that the subsequent pressure changes were essentially enzymatic in character. Decarboxylation and even deamination could be expected under these conditions. In either process, bases

TABLE 28
ANALYSES AND QUOTIENTS OF TR. LEWISI GROWN ON BLOOD AGAR

Experiment No.	1	2	3	4
No. tubes.....	2	2	2	2
No. days.....	7	11	14	28
Net air volume.....	1617.35	2016.35	2065	2052.12
Barometer.....	748	741	739	757
Temperature, C.	30	31	31	31
Corr. obs. man.	-1.0	-2.0	-4.1	-5.8
Calc. real man.	-0.8	-2.1	-4.4	-6.5
Analyses				
CO ₂	0.32	1.024	2.908	4.74
O ₂	20.55	19.701	17.552	15.50
N ₂	79.13	79.275	79.54	79.76
	100.00	100.000	100.000	100.00
Corr. analyses*				
CO ₂	0.32	1.021	2.889	4.696
O ₂	20.52	19.642	17.441	15.358
N ₂	79.04	79.04	79.04	79.04
	99.88	99.703	99.37	99.094
C c. at 0 degree, 760 mm.				
Dissolved CO ₂	0.16	0.25	4.23	5.10
Gaseous CO ₂	3.98	16.98	48.89	82.75
Total.....	4.14	17.23	53.12	87.85
O ₂ loss.....	5.629	22.25	59.66	98.82
Quotients	Average			
Apparent resp.	0.822	0.763	0.851	0.867
Real resp.	0.783	0.707	0.819	0.837
Corr. real resp.	0.822	0.735	0.890	0.888
CO ₂	0.050	0.040	0.086	0.061

* The initial gas content was assumed to be that of pure air, viz., CO₂, 0.03; O₂, 20.93; and N₂, 79.04.

would be produced, and hence gradual absorption of CO₂ would take place, resulting in a slow rise in the negative pressure. Attention may be called to the close agreement of the observed and calculated manometric readings.

The analytical values multiplied by the respective nitrogen factor gave the corrected analyses, from which the percentage of real CO₂ gain, and of the real O₂ loss were deduced. The net air volume in the jar was reduced to standard conditions, i.e., dry, at 0 degree and 760 mm., and from this value, the amounts of gaseous CO₂ made, and of O₂ lost were determined in c.c.

As indicated in the footnote to the table, it was assumed that the initial gas content was that of pure air. Actual control analyses of the air in the jar at the beginning of each experiment were made, and the results were within the limits of experimental error ($\pm .03$), and for that reason it was believed that the calculations should be based on the standard value.

It is important to note the progressive increase in the amount of O_2 consumed. At the end of 7 days, only 5.6 c.c. were utilized, whereas in 28 days the loss rose to 98.9 c.c. Similarly, the gaseous CO_2 increased from 3.9 to 82.7 c.c.

The increase in the amount of dissolved CO_2 , from 0.1 to 5.1 c.c., is especially to be observed. The latter value is greatly in excess of the amount that could be held in true solution under the partial pressure of CO_2 present. It is indicative, therefore, of the presence of bases resulting, as has been mentioned, either by decarboxylation or deamination or both.

Attention may next be directed to the respiratory quotients. It will be seen at a glance that the corrected real quotients, because of the dissolved CO_2 , were higher than the real quotients and even exceeded the apparent quotients (exper. 3 and 4). On the other hand, the real quotients were lower than the apparent quotients as a necessary consequence of the mode of calculation. Deserving of special mention is the fact that the quotients progressively increased with the duration of the experiment. This was capable of only one interpretation, namely, that additional CO_2 was being made, and that by the process of decarboxylation.

The corrected real quotient rose from 0.735 to 0.888. The question may well be asked as to which of these represents the actual respiratory change of the organism. Without doubt, the truest values are those which are obtained in the experiments in which active multiplication is still taking place. From this point of view, the corrected real quotient for *Tr. lewisi*, when growing on blood agar, should approximate the average of the values obtained on the 7th and 11th days; namely, 0.754. The average of the 4 determinations given in the table was 0.822.

Results with L. tropica.—The 4 experiments with this organism extended over 7, 11, 21 and 28 days, respectively, and are summarized in table 29.

It should be pointed out, that since *L. tropica* grows more rapidly than *Tr. lewisi*, the gas changes in the jars were more marked, especially during the first 7-11 days. Thus, the analysis on the 7th day revealed a gain in gaseous CO₂ of 24.4 c.c., as against 3.9 c.c. made

TABLE 29
ANALYSES AND QUOTIENTS OF *L. TROPICA* GROWN ON BLOOD AGAR

Experiment No.	1	2	3	4
No. tubes.....	2	2	2	2
No. days.....	7	11	21	28
Net air volume.....	2020.41	2054.12	2127	2014.41
Barometer.....	748	741	752	746
Temperature, C.	30	31	31	31
Corr. obs. man.	-2.0	-3.0	-3.4	-4.0
Calc. real man.	-2.4	-3.7	-3.9	-4.7
Analyses				
CO ₂	1.47	2.059	2.92	3.90
O ₂	19.22	18.488	17.61	16.53
N ₂	79.31	79.453	79.47	79.57
	100.00	100.000	100.00	100.00
Corr. analyses*				
CO ₂	1.46	2.048	2.90	3.874
O ₂	19.15	18.400	17.51	16.419
N ₂	79.04	79.04	79.04	79.04
	99.65	99.488	99.45	99.333
C c. at 0 degree, 760 mm.				
Dissolved CO ₂	1.09	2.215	4.23	4.23
Gaseous CO ₂	24.43	34.44	52.86	64.73
Total.....	25.52	36.655	57.09	68.96
O ₂ loss.....	30.42	43.18	62.99	75.965
Quotients	Average			
Apparent resp.	0.855	0.842	0.830	0.879
Real resp.	0.822	0.803	0.797	0.852
Corr. real resp.	0.875	0.839	0.848	0.906
CO ₂	0.063	0.044	0.064	0.065

* See footnote to table 28.

by *Tr. lewisi* in the same time. With such active respiratory change, it was to be inferred that protein cleavage would likewise be more evident. Actually, the determination of the dissolved CO₂ showed the presence of 1.1 c.c. as against 0.16 in the case of *Tr. lewisi*.

It will be seen from table 29 that the quotients increased in value with the duration of the experiment, rising from 0.839 to 0.907, the average being 0.875. These values were clearly higher than would be expected for direct protein combustion. Significant is the fact that the average for the corrected real quotients, on the 7th and 11th day, was 0.843 as against 0.754 of the slower growing *Tr. lewisi*. This marked difference may be due to excess of CO₂ production by decarboxylation.

Possibly, determinations of the quotient on the 3rd or 5th day might give a lower value which would approximate 0.754, that obtained for *Tr. lewisi*. However, it is well to bear in mind that, in air, the rapidly growing *L. tropica*, unlike *Tr. lewisi*, produces a marked change in the color of the blood agar. This reaction may be expressive of early and intense decarboxylation changes, and, if so, it may be difficult to exclude the latter.

TABLE 30
ANALYSES AND QUOTIENTS OF *Tr. lewisi* GROWN ON GLUCOSE BLOOD AGAR

Experiment No.	1	2	3
No. tubes.....	2	2	2
No. days.....	7	14	21
Net air volume.....	2016.77	1782.67	2206.8
Barometer.....	754	754	754
Temperature, C.	31	31	31
Corr. obs. man.	0.0	0.0	0.0
Calc. real man.	-0.6	-1.1	-0.5
Analyses			
CO ₂	1.11	2.934	1.446
O ₂	19.79	17.907	19.427
N ₂	79.10	79.159	79.127
	100.00	100.000	100.000
Corr. analyses*			
CO ₂	1.10	2.93	1.444
O ₂	19.77	17.88	19.406
N ₂	79.04	79.04	79.04
	99.91	99.85	99.890
C c. at 0 degree, 760 mm.			
Dissolved CO ₂	0.23	0.10	0.00
Gaseous CO ₂	18.34	43.93	26.52
Total CO ₂	18.57	44.03	26.52
O ₂ loss.....	18.89	46.20	28.59
Quotients	Average		
Apparent resp. ...	0.950	0.947	0.942
Real resp.	0.933	0.922	0.950
Corr. real resp. ...	0.938	0.934	0.953
CO ₂	0.004	0.012	0.002

* See footnote to table 28.

On the other hand, the energetic respiration of the organism may involve the combustion of substances which *Tr. lewisi* was not able to utilize. While, in general, it is to be expected that for a given medium the quotient will be constant, irrespective of the kind of organism at work, still, in view of the complexity of such a medium, selective combustion is possible and should be borne in mind.

In view of the foregoing results obtained with blood agar, it was desirable to determine the respiratory quotients for these organisms

when grown on the same medium to which 2% of glucose had been added. The method of procedure was otherwise the same as that given in connection with table 28.

Corrected Real Respiratory Quotient of Tr. lewisi on Glucose Blood Agar.—The results obtained in 3 experiments with this medium will be found in table 30. They should be carefully compared with those given in table 28. The gas exchange in exper. 3 was less than in the others, due without doubt to a poor inoculation, and hence to a more feeble growth.

A rather striking difference, to which attention should be directed at the outset, is to be seen in the amount of dissolved CO_2 . After allowing for the CO_2 present in the uninoculated control tube, the amount of the dissolved CO_2 was practically negligible, being 0.23, 0.10 and 0.0, respectively. By contrast, the results in table 28 show a progressive increase from 0.16 to 5.10 c.c. Clearly, the presence of glucose appears to prevent the decarboxylation, if not deaminization, which is so evident in the tests with plain blood agar. It may be regarded as a new expression of the so-called protein-sparing action of carbohydrates.

At the end of 7 days the O_2 loss (19.9 c.c.) was much greater than at the corresponding time in cultures grown on blood agar (5.6 c.c.). On the other hand, at the close of 14 days, it was inferior, being 46.2 as against 59.6.

Relatively, the amount of CO_2 produced on the glucose medium was higher, and this is particularly evident on comparison of the respiratory quotients. On account of the minimal amounts of dissolved CO_2 , there is much less difference between the values found of the corresponding real and the corrected real quotients than is the case in table 28. Moreover, there is little or no progressive increase in the values of the quotients according to the duration of the experiment, which fact supports the conclusion arrived at above, namely, the absence of decarboxylation.

On the 7th day, the corrected real respiratory quotient was found to be 0.934 as against 0.735 obtained with the blood agar medium. The average for the 3 experiments was 0.938.

It is evident from the value just given that 1, the theoretical quotient for glucose, was not realized. It merely goes to show that *Tr. lewisi* when growing on glucose blood agar does not obtain all of the requisite energy from glucose. If it did, then without doubt the quotient would have been 1, as has been shown to be the case for

B. tuberculosis, in Part II. It is evident, therefore, that in addition to glucose, it also utilizes some protein as a source of energy, and as a result the quotient is depressed. This depression is such as might be expected from the combustion of 3 parts of glucose and 1 part of protein, assuming the quotient of the latter to be 0.73.

TABLE 31
ANALYSES AND QUOTIENTS OF *L. TROPICA* GROWN ON GLUCOSE BLOOD AGAR

Experiment No.	1	2	3
No. tubes.....	2	2	2
No. days.....	7	14	21
Net air volume.....	2112.41	1591.07	2158.35
Barometer.....	754	754	754
Temperature, C.	31	31	31
Corr. obs. man.	0.0	0.0	0.0
Calc. real man.	-1.58	-3.16	-3.58
Analyses			
CO ₂	2.748	7.200	6.49
O ₂	18.042	13.411	14.09
N ₂	79.210	79.389	79.42
	100.000	100.000	100.00
Corr. analyses*			
CO ₂	2.74	7.168	6.459
O ₂	18.00	13.352	14.022
N ₂	79.04	79.04	79.04
	99.78	99.560	99.521
C c. at 0 degree, 760 mm.			
Dissolved CO ₂	1.23	1.73	1.83
Gaseous CO ₂	48.64	96.23	117.69
Total CO ₂	49.87	97.96	119.52
O ₂ loss.....	52.55	102.16	126.24
Quotients	Average		
Apparent resp. ...	0.946	0.941	0.944
Real resp.	0.932	0.925	0.930
Corr. real resp. ...	0.951	0.949	0.946
CO ₂	0.019	0.025	0.015

* See footnote to table 28.

Corrected Real Respiratory Quotient of L. tropica on Glucose Blood Agar.—A set of 3 experiments, similar to those just given for *Tr. lewisi*, were made with *L. tropica*, and the results obtained are given in table 31.

On comparing this with table 29, several interesting facts will be evident. Thus, the amount of dissolved CO₂ ranged from 1.2 to 1.8, while, for the blood agar medium, it rose from 1.1 to 4.2. It would appear that with the active and rapidly growing *L. tropica*, the carboxylase action was only partly suppressed as compared with the effect seen in the experiments with *Tr. lewisi* (table 30).

The O_2 loss in 7 to 21 days rose from 52.5 to 126.2 as compared with 30.4 to 62.9 on plain blood agar. Hence, in 21 days, the O_2 consumption, per tube, was 63 c.c. for glucose blood agar, and 31.5 c.c. for plain blood agar.

The CO_2 gain, similarly, increased from 49.8 to 119.5, whereas on plain blood agar it went from 25.5 to 57.1. On the 21st day, per tube, the CO_2 gain was approximately 60 and 28.5 c.c., respectively.

The corrected real respiratory quotients obtained in the 3 experiments showed a satisfactory agreement, varying only from 0.946 to 0.959, with an average of 0.951. The average for *Tr. lewisi* on the same medium was 0.938, the difference being due to the relative amounts of dissolved CO_2 .

As in the case of *Tr. lewisi*, the respiratory quotient of 1 was not realized in these tests with *L. tropica*. The presence of glucose did not completely suppress the combustion of proteins, or the action of carboxylase; hence, the quotient of 0.95 instead of 1.0, as was expected.

In arriving at the corrected real respiratory quotients given in the preceding tables, the utmost care was taken to obtain true values for the dissolved CO_2 . It should be pointed out that these determinations were the least accurate, since they involved the removal of the gas from the finely comminuted medium. Possibly, better values could be obtained if the medium could be completely liquefied, but this was impossible with blood mediums. The amount of CO_2 taken up by the blood agar medium was very large, especially so when the incubation was carried on for 14 days or more, as will be seen on reference to tables 28 and 29.

It must be remembered in this connection that, in the foregoing experiments, anaerobic jars of about 2,000 c.c. capacity were used. Consequently, the concentration of the free CO_2 could at no time reach that which was found when *h*-tubes were employed. In the latter, the O_2 had practically disappeared, and about 14% of CO_2 was present. It follows, therefore, that a relatively larger amount of CO_2 should be taken up by the medium in *h*-tubes than was the case in the jar experiments. That this actually occurred will be seen on reference to table 32.

The net air volume, unreduced, in the *h*-tubes was about 100 c.c. It is to be noted that, largely because of the high percentage of CO_2 (13-15%), with one exception, the dissolved CO_2 was in excess of 5 c.c. In jar exper. 2 of table 29, of the same duration, the dissolved CO_2 was 2.2 c.c., while the gaseous CO_2 was 2.05%.

A comparison, in the case of tubes 1 and 2, of the actual volumes of dissolved and of gaseous CO_2 shows that the former was nearly one-half of the latter. The effect of this large amount of dissolved CO_2 on the calculation of the respiratory quotients was marked. Thus, while the real quotients in table 32 were about 0.15 lower than those in table 29, the corrected real quotients were more than 0.1 higher

TABLE 32

L. TROPICA ON BLOOD AGAR, IN *h*-TUBES, AT 31 C.: SHOWING EFFECT OF RUBBER STOPPERS ON CONTENT OF CO_2 AND ON THE QUOTIENT VALUES

Closed with.....	Ground Glass Caps					Rubber Stoppers				
Exper. No.	1		2			2a				
Tube No.	1	2	3	4	5c	6	7	8	9c	10c
Duration, hrs.	240	264	240	264	240	240	240	264	12	264
Corr. obs. man.	-43	-44.2	-44.2	-46.1	0	-63.6	-61.9	-57.4	0	-7
Calc. real man.	-43.2	-46.6	-51.2	-51.6	-63.6	-61.9	-61.3
Analyses										
CO ₂	15.447	15.456	14.86	14.80	1.16	13.24	13.46	13.54	0.286	1.25
O ₂	0.0	0.0	0.0	0.0	19.61	0.0	0.0	0.0	20.596	19.27
N ₂	84.553	84.544	85.14	85.20	79.23	86.76	86.54	86.46	79.118	79.48
	100.000	100.000	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
Corr. analyses*										
CO ₂	14.44	14.45	13.795	13.730	12.062	12.293	12.378
O ₂	0.0	0.0	0.0	0.0	0.0	0.0	0.0
N ₂	79.04	79.04	79.04	79.04	79.04	79.04	79.04
	93.48	93.49	92.835	92.77	91.102	91.333	91.418
C c. at 0 degree, 760 mm.										
Dissolved CO ₂	5.120	5.431	9.23	5.68	0.87	3.89	5.34	5.28	0.78
Gaseous CO ₂	11.73	12.176	11.5	11.37	9.19	8.91	9.26
Total CO ₂	16.85	17.607	20.73	17.05	13.08	14.25	14.54
O ₂ loss.....	17.037	17.673	17.49	17.37	15.99	15.21	15.69
Quotients										
Apparent resp. ..	0.736	0.737	0.709	0.705	0.631	0.641	0.645
Real resp.	0.688	0.689	0.658	0.654	0.575	0.586	0.590
Corr. real resp. ..	0.989	0.996	1.180	0.981	0.818	0.937	0.927
CO ₂	0.436	0.446	0.800	0.499	0.423	0.579	0.570

* See footnote to table 28.

5c, 9c and 10c were uninoculated controls.

The dissolved CO_2 in nos. 1 and 2 represents the value found less 0.387, the amount present in a control tube at start of experiment.

In nos. 3, 4, 6, 7 and 8 the deduction was 0.78, which was the amount present in control 10 at the close of the experiment.

than in the latter table, and actually approximated 1. Incidentally, attention should be called to the high values for the CO_2 quotients, which were much higher than those given in table 29.

Table 32 is primarily intended to bring out the fact that an appreciable loss of CO_2 resulted when rubber stoppered *h*-tubes were used. These were always attached to the manometers by means of no. 3 or 4 rubber stoppers treated with glycerol, and the latter, as was shown

in table 16, will absorb CO_2 , thus reducing the amount of CO_2 over the culture. It was desirable to ascertain how great was the loss of CO_2 due to the rubber stopper. For this purpose, *h*-tubes were provided with ground glass caps, such as are shown in fig. 2*B*, Part I. After inoculation, these tubes (nos. 1-5) were attached to the manometers by means of no. 25 rubber stoppers, thus making glass

TABLE 33
AVERAGES OF DETERMINATIONS OF RESPIRATORY QUOTIENTS

Respiratory quotient.....	L. tropica		Tr. Lewisi	
	Real	Corr. Real	Real	Corr. Real
Blood agar				
<i>h</i> -tube, rubber stopper.....	0.648 (19)	0.658 (13)
Glass cap.....	0.672 (4)	0.992 (2)
Jars.....	0.822 (4)	0.875 (4)	0.783 (4)	0.822 (4)
Glucose blood agar				
<i>h</i> -tube, rubber stopper.....	0.800 (4)	0.838 (4)
<i>h</i> -tube rubber stopper.....	0.858 (5)	0.935 (5)
Jars.....	0.932 (3)	0.951 (3)	0.933 (3)	0.938 (3)

The figures in parentheses indicate the number of tests which were averaged.

TABLE 34
GAS CHANGES, PER TUBE OF TR. LEWISI AND L. TROPICA GROWN ON BLOOD AGAR AND ON GLUCOSE BLOOD AGAR (TABLES 28-31)

Medium.....	Blood Agar				Glucose Blood Agar		
	7	11	14	28	7	14	21
Days.....							
Tr. lewisi							
At 0 degree and 760 mm.							
CO_2 made.....	2.07	8.61	26.51	43.92	9.28	22.01	13.26
O_2 lost.....	2.81	11.12	29.83	49.41	9.94	23.01	14.30
At 30 degrees and 750 mm.							
CO_2 made.....	2.43	10.11	31.14	51.58	10.90	25.85	15.57
O_2 lost.....	3.30	13.06	34.92	58.03	11.67	27.02	16.79
Air volume.....	15.77	62.40	167.40	277.28	55.78	129.13	80.25
Percentage CO_2	15.38	16.20	18.63	18.58	19.55	19.94	19.40
Days.....	7	11	21	28	7	14	21
L. tropica							
At 0 degree and 760 mm.							
CO_2 made.....	12.76	18.32	28.54	34.48	24.93	48.98	59.76
O_2 lost.....	15.21	21.59	31.49	37.93	26.27	51.08	63.12
At 30 degrees and 750 mm.							
CO_2 made.....	14.99	21.52	33.52	40.50	29.28	57.53	70.19
O_2 lost.....	17.86	25.36	36.98	44.55	31.18	60.00	74.14
Air volume.....	85.35	121.16	176.72	212.85	147.42	286.65	354.22
Percentage CO_2	17.56	17.74	18.96	18.96	19.86	20.07	19.79

to glass connections. Tubes 6 to 10 were attached in the usual way, as in all of the previous experiments. Experiments 2 and 2*a* were made at the same time, and the results are, therefore, directly comparable. Experiment 1, it should be added, was made at a different time.

The observed manometric readings of the tubes which were provided with rubber stoppers (nos. 6-8) were about 15 mm. higher

than those with glass caps (nos. 1-4). This in itself indicated a loss of about 2% of CO_2 . The analyses confirmed this conclusion, since the tubes with rubber stoppers had only about 13%, while the glass-capped tubes had about 15% of CO_2 . The average real loss as deduced from the corrected analyses for tubes 6-8 was 8.7, while that for tubes 1-4 was 6.3, the difference being due to the actual loss of CO_2 by solution in the rubber stopper.

The effect of this loss of CO_2 on the respiratory quotients was considerable. The average for the apparent quotients of the glass-capped tubes was 0.722, as against 0.639 of those with rubber stoppers. Similarly, the real quotients average 0.672 and 0.584, respectively. The corrected real quotients for nos. 1 and 2, because of the relatively large amount of dissolved CO_2 , averaged 0.993, whereas for the jar experiments in table 29, they averaged 0.875.

Another source of error is the uncertainty of the exact composition of the air which is present in the culture tubes at the start of an experiment. The air is subject to variation in composition, even in a given set of tubes. This error is minimized when jars instead of tubes are used as the respiratory chambers.

The value of the real respiratory quotient may be depressed, as has been shown above, by a loss of CO_2 . This quotient may also be lowered as the result of direct absorption of some O_2 by the reducing products of the organism. On the other hand, the production of CO_2 by carboxylase action would cause an increase in the quotient.

In table 33 are given the averages for the real and corrected real quotients as recorded in all of the previous tables. It will be seen at a glance that the real quotient was lowest when the *h*-tubes were used in the ordinary way. When used with glass caps, the values were appreciably higher but still inferior to those obtained with jars. Again, the corrected real quotients for *L. tropica* were higher than those for *Tr. lewisi*.

It is evident from the table that there is an appreciable error when the *h*-tubes are attached to the manometers in the usual way. The results are only approximate because of the loss of CO_2 . This loss can be reduced by using either glass-capped *h*-tubes, or glass-stoppered side-arm tubes. Obviously, the quotients obtained with Novy jars are free of this and other errors, incidental to the use of tubes, and are therefore to be accepted as the true values.

Gas Changes per Tube of Tr. lewisi and L. tropica.—From the jar experiments given in tables 28-31, it is possible to deduce values which

represent the gas changes brought about by the developing culture within a single tube. The instructive results thus obtained are brought together in table 34.

The table gives, in the first place, the number of c.c. of CO₂ made and of O₂ consumed by a culture at the end of the several periods of incubation. These results express the values under standard conditions, i. e., 0 degree and 760 mm., dry.

In order to show the corresponding values, under the conditions of cultivation, the foregoing results were recalculated to 30 C. and 750 mm. The formula for this conversion was given in Part II and for the conditions mentioned it becomes

$$V = V_0 \times 1.17455.$$

Multiplying the gas volumes, under standard conditions, by this factor gave the values for CO₂ made and O₂ consumed at 30 C. and 750 mm.

Further, to obtain a definite idea of the volume of air utilized by a culture, the number of c.c. of O₂ consumed under standard conditions was multiplied by 5.6118. This value was arrived at by multiplying the foregoing factor (1.17455) by 100 and dividing by 20.93, the oxygen content of pure air.

It will be seen from the table that the gas changes due to *L. tropica* on blood agar, on the 7th day, were more than 5 times those of *Tr. lewisi*. On the 11th day, they were twice as large, but after that the difference became less, so that seemingly by the 14th day they became inferior to those of *Tr. lewisi*. It is worth while to point out that about 100 c.c. of air were needed to obtain good rich cultures of either organism.

The gas changes on glucose blood agar on the 7th day were greater than those on the usual medium. With *Tr. lewisi*, the difference was nearly 4-fold, while with *L. tropica* it was only about double.

The table also contains values which represent the percentage of CO₂ which would be found in the tube provided no CO₂ was taken up by the medium. These values were based on the corrected real respiratory quotients, as given in tables 28-31, and on the assumption that all of the O₂ present in the air (20.93) was consumed, the formula being:

$$\text{Percentage of CO}_2 \text{ produced} = \text{corrected real} \\ \text{quotient} \times \text{percentage of O}_2 \text{ consumed (20.93)}.$$

It has been shown (e. g., table 32) that the gas in a culture tube, on analysis, contained 13-15% of CO₂ when all of the O₂ had been consumed. Obviously, these figures did not represent the total yield,

since a considerable volume of CO_2 was taken up by the medium. The real percentage of CO_2 produced, as calculated in table 34, ranged from 15-19% for blood agar. The higher values, in this case, as pointed out heretofore, were probably due to secondary CO_2 production. The values for the glucose blood agar were more uniform, and corresponded closely to what was to be expected for the respiratory quotient of glucose.

SUMMARY

For the first time, a detailed study of the respiration of pure cultures of two well-known protozoa is presented.

The results show that the gas exchange is the same as in the case of bacteria, although in this paper no direct comparison is made. That this is true will be seen on reference to Part II, which deals with the respiration of *B. tuberculosis*.

When grown in tubes which have a capacity of about 100 c.c. of air, *L. tropica* consumed all of the O_2 in about 144 hours; whereas *Tr. lewisi* required about twice that time. The yield of CO_2 was about the same with both organisms, amounting to about 15%, uncorrected for the dissolved gas.

When grown in jars which have a capacity of about 2,000 c.c., 2 tubes of *L. tropica*, in 28 days, produced 68.96 c.c. of CO_2 and consumed 75.96 c.c. of O_2 . Similarly, 2 tubes of *Tr. lewisi* produced 87.85 c.c. of CO_2 and consumed 98.82 c.c. of O_2 . These values are calculated for standard conditions, 0 degree and 760 mm.

The manometer is an excellent index of the growth of these organisms. The readings obtained vary with the O_2 concentration; thus, when air was present in the culture tube, the maximal negative pressure was about 50 mm. In 50% O_2 , *L. tropica* gave readings as high as -157 mm. The cessation of growth was indicated by a constancy in the readings. When glucose was present in the medium, the manometric reading was less than when it was absent. When proper precautions were taken, the calculated manometric reading, based on the real loss, approximated closely the corrected observed readings.

Oxygen pressures of varying amounts were used, and the effects noted. *Tr. lewisi* was more sensitive to increasing partial pressures of O_2 than *L. tropica*. The germs were not killed by high tensions of O_2 but their growth was inhibited, since subsequent incubation in air gave rich cultures.

A concentration of 50% O_2 was the critical point for the growth of *L. tropica*. It was able to consume all of this O_2 in the tubes used,

and returned nearly 34% of CO_2 . At 60% of O_2 concentration, the growth was inhibited, as shown by the lower manometric readings and by the lessened O_2 consumption and CO_2 production.

The parasites were found to be obligative aerobes. They required free O_2 for growth and multiplication. Atmospheres of N_2 or H_2 , in which O_2 was absent, inhibited growth. These elements, however, were not toxic. CO_2 in concentrations greater than 20% was toxic for *Tr. lewisi*, even when an excess of O_2 was present. *L. tropica* was less sensitive to CO_2 , and partial pressures greater than 30% were necessary to produce injurious effects.

CO_2 was not essential to the life of these organisms. In the presence of alkali, growth was obtained, but the cultures, instead of forming a more or less uniform mass over the surface of the medium, showed only small isolated colonies. Desiccation of the surface appeared to be the cause of such poor growth.

The presence of defibrinated blood or serum was essential for the growth of the germs. No difference in the gas relations was noted by the substitution of serum for the blood.

The value of the respiratory quotient increased with the duration of the experiment, and this was interpreted as being due to secondary changes in the medium. The true respiratory quotient is obtainable when the culture is young and before such secondary changes can affect the result.

With blood agar medium, the corrected real respiratory quotient for *L. tropica* was found on the 7th day to be 0.839, and on the 28th day 0.907. Similarly, *Tr. lewisi* gave 0.735 on the 7th day and 0.888 on the 28th day. The average of 4 determinations (7-28 days) for *L. tropica* was 0.875, while for *Tr. lewisi* it was 0.822.

When glucose was present in the medium, the corrected real respiratory quotient was found to average 0.951 for *L. tropica* and 0.938 for *Tr. lewisi*.

The amount of CO_2 taken up by the medium was considerably less when glucose was present, indicating a protein-sparing action.

The large amount of CO_2 taken up by the plain blood agar medium indicated the formation of basic products either by deaminization or by decarboxylation, or both.

Per tube of culture, in 28 days, *Tr. lewisi* consumed all of the O_2 in 277 c.c. of air, under ordinary conditions, i. e. 30 C. and 750 mm.; *L. tropica*, in the same time, utilized 212 c.c. of air. The latter on glucose blood agar, in 21 days, consumed the O_2 in 354 c.c. of air.

THE DIFFERENTIATION OF THE PARATYPHOID ENTERITIDIS GROUP

IX. STRAINS FROM VARIOUS MAMMALIAN HOSTS

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Bacteria of this group have been reported most frequently in connection with human and with porcine infections, but more or less specialized members of the group are also associated with certain pathologic conditions in other mammals. Infectious abortion of mares, a similar disease of guinea-pigs, "pseudotuberculosis" of guinea-pigs, septicemia and infectious diarrhea of calves and mouse typhoid are among the more definitely described mammalian paratyphoid infections. A number of epidemics of diarrheal diseases in white rats¹ and in rabbits² have also been reported.

Equine Strains.—Organisms of the paratyphoid enteritidis group occurring in outbreaks of abortion in mares were apparently first described by Theobald Smith in 1893 (Pennsylvania) and later by Lignières (France and Argentine, 1897), Good (Kentucky, 1911), van Heelsbergen and de Jong (Holland, 1912-1914), Meyer and Boerner, (Pennsylvania, 1913), Schofield (Canada, 1914), Murray (Iowa, 1919), Kii, Sato, Nakamura, Taguchi (Japan, 1923), and others.³ These organisms are substantially identical according to most observers.⁴ The name *B. abortivus equinus* (later corrected to *B. abortivo-equinus*) was given to this bacillus by Good and Corbett (April 26, 1913),⁵ and appears to have priority, although the name *B. abortusequi* (properly *abortus-equi*) proposed by Meyer and Boerner (July 18, 1913),⁶ about the same time has come into more general use. The equine type is not recognized as a distinct member of the group by Winslow, Kligler and Rothberg in

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¹ Cannon: Jour. Infect. Dis., 1920, 26, p. 402.

² Litch and Meyer: Ibid., 1921, 28, p. 27.

³ Branford and Doyle (Abst. Bacteriol., 1922, 6, p. 366) in India have isolated from contagious abortion in pony and donkey mares organisms that could not be carefully studied for lack of facilities, but seemed to be the same as those found in contagious abortion in horses.

⁴ Meyer and Boerner: J. Med. Res., 1913, 29, p. 325. Good and Smith: Bull. 204, Ky. Agri. Expt. Station, 1916. Murray: J. Infect. Dis., 1919, 25, p. 341. Fitch and Billings: J. Bacteriol., 1920, 5, p. 469. Kii, Sato, Nakamura, Taguchi, Scientif. Rept. Gov't. Inst. for Infect. Dis., 1923, 2, p. 135.

⁵ Jour. Infect. Dis., 1913, 13, p. 53.

⁶ Jour. Med. Res., 1913, 29, p. 325.

their extensive comparative study.⁷ According to the nomenclature of the Society of American Bacteriologists,⁸ this organism would be placed in the genus *Bacterium*.

An organism of this same group has been isolated from the joints and blood of certain foals suffering from "joint-ill" or septic arthritis,⁹ and has been generally regarded as identical with *B. abortivo-equinus*.¹⁰ Schofield thinks that the evidence points to the foal being frequently infected before birth through the umbilicus. While there is no doubt of the general group relationship of the organisms from joint-ill and those from infectious abortion, no comprehensive cultural and serologic comparison seems to have been made, so that exact identity is not established.

The infectious nature of certain outbreaks of abortion in mares was recognized in various parts of Europe in the first part of the 19th century and perhaps earlier (Lautenbach, van Heelsbergen). Infectious abortion in mares does not appear to have attracted attention in the United States before 1886, when it suddenly became conspicuous among draft horses in some parts of the Mississippi Valley.¹¹ This may indicate that the specific organism was imported into this country. There is little or no evidence that bacilli of this group occur in normal horses. V. A. Moore, as reported by Smith (1893), noted that the type of bacillus found by Smith in aborting mares was absent from the genital passages of four healthy nonpregnant mares and one healthy pregnant mare.

The differences between the bacilli isolated from infectious abortion in mares and other paratyphoid bacilli are both cultural and serologic. In agglutinative reactions, the equine strains constitute a distinct group.⁴

Lautenbach¹² states that the bacillus isolated by him was nearest to *B. paratyphosus* A in its agglutinative reaction, but this observation stands alone.

On slanted agar the growth is membranous, dry and brittle, (T. Smith, Meyer and Boerner, Good and Smith, Murray, Kii, Sato, Nakamura and Taguchi). The general fermentation reactions are like those

⁷ Jour. Bacteriol., 1919, 4, p. 429.

⁸ Ibid., 1920, 5, p. 191.

⁹ By no means all cases of joint-ill are accompanied by this organism. McFadyean and Edwards (J. Comp. Path. & Therap., 1919, 32, p. 42) found it in but 2 out of 37 cases examined; in both instances the foals were from a stud in which mares had recently aborted. Streptococci were present in the majority of joint-ill cases.

¹⁰ Meyer and Boerner: J. Med. Res., 1913, 29, p. 325. Good and Smith: J. Infect. Dis., 1914, 15, p. 397.

¹¹ Williams: Veterinary Obstetrics, p. 478.

¹² Centralbl. f. Bacteriol., I, O., 1913, 71, p. 349.

of the other members of the group (Meyer and Boerner, Fitch and Billings).

So far as can be gathered from the descriptions given of cultural characteristics and agglutinative reactions, most observers have found in these cases of equine abortion paratyphoid bacilli of rather distinctive character agreeing among themselves and forming a group somewhat apart from the paratyphoid bacilli isolated from the bodies of other animals. Gminder,¹³ however, in a study of 33 cultures from aborting mares found 1 strain agglutinatively like *B. enteritidis* and several others (8?) that showed high agglutination with *B. paratyphosus* B serum. Differential cultural reactions were not recorded, and no serum absorption tests were made. At least 24 of his 33 cultures were definitely of the *B. abortivo-equinus* type.

The strains that I have had under observation number 16, 14 of these from the aborted fetus or the uterine exudate of aborting mares in Kentucky (8 strains, kindness of Prof. E. S. Good) and Pennsylvania (6 strains, kindness of Drs. K. F. Meyer and F. Boerner); a 15th strain is of European origin (Holland) and was received by Dr. Meyer from van Heelsbergen in 1914. The 16th strain was received from the Lister Institute collection and was apparently derived from fetal material from an ass in an epidemic of abortion in the Belgian Congo.¹⁴

All 16 strains have behaved alike in their cultural and their serologic reactions. The dry, membranous growth on agar noted by several observers has been manifest on every transfer and would almost serve in itself to mark off *B. abortivo-equinus* from all the other members of the paratyphoid group.

Repeated serologic tests have been made. In one typical instance, an abortivo-equinus serum with a titer of 1:10,000 agglutinated all the organisms of this series, practically to the titer limit, while it had no agglutinative action in 1:250 dilution on *B. paratyphosus* A (17 strains), *B. paratyphosus* B (31 strains), *B. suipestifer* (38 strains) and *B. enteritidis* (10 strains). Conversely, the serums produced by certain members of these other groups with titers ranging from 5,000 to 10,000 have no agglutinative action on the strains of *B. abortivo-equinus* in dilutions of 1:250. Certain other strains of *B. paratyphoid* and of *B. enteritidis*, however, have yielded serums which had a definite agglutinative action (over 1:250)—although never to the titer limit—

¹³ Arb. a. d. Reichsndtsamt., 1920, 52, p. 113.

¹⁴ Brugnotte, Compt. rend. Soc. de biol., 1919, 82, p. 954.

on the abortivo-equinus group. Absorption tests showed the reaction to be due in every case to group and not to specific agglutinins. Two serums of the suipestifer group and one of the C group have also been tested and have never agglutinated the abortivo-equinus strains.

There are also certain cultural distinctions that do not seem to have been noted previously. As I have pointed out elsewhere,¹⁵ arabinose and dulcitol fermentation and blackening of lead acetate medium distinguish *B. paratyphosus* B from the closely allied *B. suipestifer*. With respect to arabinose and dulcitol fermentation, *B. abortivo-equinus* resembles *B. paratyphosus* B, both these carbohydrates being fermented promptly and vigorously by all 16 strains. Lead acetate medium, on the other hand, is not blackened, so that in this respect the abortivo-equinus strains stand closer to *B. suipestifer* than to *B. paratyphosus* B. Two other fermentation reactions are important. Trehalose has been shown by Koser¹⁶ to be of differential value for other members of the group, *B. suipestifer* being unable to attack this disaccharide, whereas *B. paratyphosus* A, *B. paratyphosus* B, and *B. enteritidis* ferment it with acid and gas production. Trehalose is fermented by all 16 *B. abortivo-equinus* strains, which in this character resemble *B. paratyphosus* B, and differ from *B. suipestifer*. Finally, inosite is not fermented by any of the abortivo-equinus strains. This negative result is like the *B. suipestifer* reactions (also negative) and different from the *B. paratyphosus* B reactions (mostly inosite positive).

In tabular form, the results are as follows:

B. abortivo-equinus (16 strains) serologically distinct from all other members of the paratyphoid-enteritidis group.

Cultural reactions:

<i>Like B. paratyphosus</i> B	<i>Like B. suipestifer</i>
Rapid arabinose fermentation	No blackening lead acetate
Rapid dulcitol fermentation	No inosite fermentation
Trehalose fermentation	

Whether other bacteria of this group sometimes occur in normal or diseased horses is more or less uncertain. Titze and Weichel¹⁷ report finding in a normal horse a bacillus resembling in all respects the bacillus of calf diarrhea, but the details given for identification are inadequate. More recently Graham, Reynolds and Hill¹⁸ have reported finding in a

¹⁵ Jour. Infect. Dis., 1917, 29, p. 467; *ibid.*, 1917, 21, p. 271.

¹⁶ *Ibid.*, 1921, 29, p. 67.

¹⁷ Arb. a. d. k. Ges., 1910, 33, p. 516.

¹⁸ Jour. Am. Vet. Med. Assn., 1920, 56, p. 378.

peracute disease of horses and mules a bacillus which they identified as *B. enteritidis*. A similar organism was isolated by them from the feces of a healthy horse. In this instance also fuller cultural details and an agglutination comparison with cultures of known characteristics would have been desirable. As has been often emphasized in these studies, the name by which a stock culture is labeled cannot always be taken as indicative of its true nature.

Rodent Strains.—Highly fatal outbreaks of acute infectious disease among laboratory guinea-pigs, mice and white rats due to bacilli of this group have been noted frequently. Löffler, who observed such an outbreak in laboratory mice in 1892, gave the name *B. typhi murium* to the organism isolated. This name has been applied somewhat indiscriminately by subsequent observers, and cultures labeled *B. typhi murium* have been found to belong in some cases to the *B. enteritidis* type and in others to the “animal paratyphoids” or *B. aertrycke* type.¹⁹ The so-called Danysz bacillus was isolated (1900) from field mice affected by a spontaneous epidemic, and, owing to its high virulence for rats, has been used for rat and mice destruction. The nature of cultures in laboratory collections labeled “*B. typhi murium*” or “Danysz bacillus” is uncertain unless these cultures are subjected to specific examination.

Besides appearing as probable causal agents in epidemics of acute disease, paratyphoid-enteritidis bacilli have been found also in the pseudotuberculosis of guinea-pigs (Theobald Smith) and in infectious endometritis of these animals (Carter’s bacillus²⁰). Several other observers²¹ have reported finding these organisms in diseased conditions in guinea-pigs, and a new name, *B. pestis caviae*, has been applied to some of the guinea-pig bacilli on the basis of agglutination differences. An earlier name for these organisms, *B. pseudotuberculosis rodentium*, seems to have been based on source rather than on the demonstration of differential cultural characters.

Rodent strains may be considered as belonging to two main types: (a) *B. enteritidis* and (b) bacilli closely related to, but agglutinatively not identical with, the human *B. paratyphosus* B type.

(a) *B. enteritidis.*—Trautman,²² who compared a number of strains isolated from spontaneous outbreaks in laboratory rats, found them to

¹⁹ Bainbridge: *J. Path. & Bacteriol.*, 1909, 13, p. 443. Krumwiede, Valentine and Kohn: *Jour. Med. Res.*, 1919, 34, p. 449.

²⁰ Smith and Reagh: *Jour. Med. Res.*, 1903, 4, p. 270.

²¹ Wherry: *Jour. Infect. Dis.*, 1908, 5, p. 519; Kirch: *Arch. f. Hyg.*, 1912-13, 78, p. 327.

²² *Ztschr. f. Hyg. u. Infektionskr.*, 1906, 54, p. 104.

be completely identical with the Danysz bacillus and *B. enteritidis*. Schern²³ also described an outbreak in rats due to *B. enteritidis*. A spontaneous laboratory epidemic among white rats in New York, apparently due to *B. enteritidis*, has been reported by Pappenheimer and Wedel.²⁴ A similar outbreak occurred among rats in this laboratory in 1919, and was reported by Cannon;²⁵ about 3 years later, after an intervening period during which no infections of this type were observed, white rats and mice in the laboratory were again affected, and *B. enteritidis* was isolated from blood and internal organs as before.²⁶ In addition to the cultures from our own laboratory outbreaks, 3 other cultures from white rats have come into my possession: no. 137 from a rat injected with a rat virus called azoa; received from Dr. W. B. Wherry, University of Cincinnati;²⁷ no. 204 obtained from the heart blood of a white rat during an epidemic in the laboratory, received from Dr. J. G. Cumming, University of California; no. 366 from spontaneous infection in a rat (multiple pulmonary abscesses), received from Lister Institute National Collection of Type Cultures. All these cultures are of the true *B. enteritidis* type.

The commercial rat viruses so far as they have been studied appear for the most part to contain *B. enteritidis*. Bainbridge,²⁸ who examined 5 rat viruses in England, found that all but one—which contained “*B. aertryck*”—contained *B. enteritidis*. Savage and Read,²⁹ who isolated *B. enteritidis* from the internal organs of wild rats, obtained in Weston-super-Mare, regarded this organism as probably derived from a Danysz virus used in slaughter houses some years before. In a later paper, Savage and White³⁰ report isolating *B. enteritidis* in 6 out of 96 rats caught in English slaughter houses. It was considered uncertain whether bacterial bait or natural infection was the origin of this strain.

Three rat viruses examined by me: “Liverpool Virus,” “Ratin,” “Imperial Virus,” all contain *B. enteritidis*. The “azoa” virus examined by Wherry³¹ was apparently of the same type. It does not seem possible to determine which of these virus cultures if any are descended from the parent Danysz organism.

²³ Arb. a. d. k. Ges., 1909, 30, p. 575.

²⁴ Jour. Infect. Dis., 1914, 14, p. 180.

²⁵ Ibid., 1920, 26, p. 402.

²⁶ Unpublished observations of E. Yuri.

²⁷ Jour. Infect. Dis., 1908, 5, p. 519.

²⁸ Jour. Path. & Bacteriol., 1909, 13, p. 445.

²⁹ Jour. Hyg., 1913, 13, p. 343.

³⁰ Ibid., 1923, 21, p. 258.

³¹ Jour. Infect. Dis., 1908, 5, p. 519.

B. enteritidis is evidently the common type in paratyphoid infection of laboratory white rats and possibly of wild rats. No established instance of rat infection with the paratyphosus B type has come to my notice. All of the rat cultures in my collection (4 from diseased rats, 4 from rat viruses) are of the *B. enteritidis* type.

B. enteritidis has also been isolated from mice. There seems little doubt that the original Danysz strain from field mice was of this type. Only one of my cultures (323) has been from mice. This culture, kindly sent me by Dr. Wherry, was isolated from mice sprayed with influenza sputum and is a typical *B. enteritidis*.³² Tenbroeck³³ says: "In my experience, most of the cultures from mice and guinea pigs belong to the enteritidis group."

Strains derived from guinea-pigs are, in part, of the *B. enteritidis* type. Of 16 guinea-pig cultures in my collection, 6 are *B. enteritidis*. Three of these were received from Dr. Wherry, who isolated them from spleen nodules in cases of pseudotuberculosis in guinea-pigs. They are typically *B. enteritidis*, and after 16 years' cultivation agree in all respects with Wherry's original description.³¹ Another culture, received through Dr. J. G. Cumming, was obtained by him from Dr. Harry Plotz as coming from an outbreak of epidemic abortion in guinea-pigs. Two cultures from Dr. R. W. Pryer of the Detroit Health Department were isolated by him from the blood of guinea-pigs that had been inoculated with suspected food material (olives). These last two strains may have been present in the material injected, but it is at least equally probable that they were originally harbored by the inoculated animal.

The reported finding of *B. enteritidis* in mice inoculated with influenza sputum (Wherry) may perhaps be accounted for similarly.

Krumwiede, Valentine and Kohn believe the *B. enteritidis* type to be less common in guinea-pigs than the paratyphosus type, but state that they have isolated both the *B. enteritidis* and "pestis caviae" types from their own laboratory animals in the same epidemic. Thomas³⁴ also found little variation in guinea-pigs dying from spontaneous infection.

B. paratyphosus B (Type Aertrycke).—I have elsewhere³⁵ shown that the paratyphoid B bacilli of rodent origin belong for the most part, if not altogether, to a particular agglutinative type which may be called

³² Ibid., 1920, 27, p. 315.

³³ Jour. Exper. Med., 1920, 32, p. 19.

³⁴ Ibid., 1924, 35, p. 407.

³⁵ Ibid., 1923, 33, p. 567.

provisionally the Aertrycke type. Krumwiede, Valentine and Kohn had earlier shown the existence of an agglutinatively separate rodent group, but were inclined to regard it as distinct from the *B. paratyphosus* B group. There are strong reasons, however, for considering the Aertrycke (rodent) and Schottmüller types as closely related members of one division.³⁵

Type Aertrycke paratyphoid bacilli sometimes have been isolated in outbreaks of disease in the guinea-pig. It is not clear from the published reports just how frequently these organisms occur. Owing to the insufficiency of the tests employed for differentiation, it is not possible, in many instances, to determine the precise nature of the organism designated by authors as a "paratyphoid" or a "Gärtner" bacillus. Holman's summary³⁶ of the recorded spontaneous infections of the guinea-pig by members of the *B. paratyphosus* group makes plain the incompleteness of many of the existing descriptions. Among the few definite instances of the isolation of type Aertrycke strains from guinea-pigs may be mentioned those reported by Krumwiede, Valentine and Kohn,¹⁹ who studied 13 strains of this type isolated from guinea-pigs, by Howell and Schultz³⁷ who observed an epizootic among laboratory animals in Chicago, and by Thomas.³⁸ It seems probable that some of the earlier outbreaks of "pseudotuberculosis" among guinea-pigs should be referred to this type, since a number of workers evidently found difficulty in identifying the organisms isolated from this disease with other members of the group; hence the special names, *B. pestis caviae*, etc., so freely given.

During the past 10 years, I have had under observation 17 strains isolated from guinea-pigs; one of these is from a guinea-pig that had been inoculated with "hog cholera virus" and is definitely of the *B. suispestifer* type; it does not need further consideration here. Six of the remaining 16 are *B. enteritidis*; the other 10 belong to the *B. paratyphosus* B, Aertrycke type. Four of the latter were received in 1916 and 1917 from Major C. L. Cole of Fort Sam Houston, Texas, and were isolated by him at various times: 2 from the organs of guinea-pigs dying after giving birth to young, 2 from guinea-pigs dying during an epidemic septicemia of the laboratory stock; 1 is the strain described by Howell and Schultz;³⁷ 1 was received from Dr. W. G. Savage and had been isolated from a spontaneous outbreak in laboratory stock;

³⁶ Jour. Med. Res., 1916, 30, p. 151.

³⁷ Jour. Infect. Dis., 1922, 30, p. 516.

³⁸ Ibid., 1924, 35, p. 407.

1 was received from the Lister Institute, where it was recorded as the cause of a guinea-pig epizootic; 2 came from Dr. R. W. Pryer of Detroit and were from guinea-pigs inoculated with material derived from seized ripe olives; 1 from Dr. S. R. Haythorn of the University of Pittsburgh, isolated from a spontaneous outbreak among laboratory guinea-pigs.³⁹

Krumwiede, Valentine and Kohn,¹⁹ in addition to the guinea-pig strains which they studied, describe 2 strains presumably from mice (labeled "typhi murium"; history unknown), 1 from a rabbit, 1 from a cat, as possessing the characters of the rodent type. The "mouse typhoid" strain used in the Rockefeller Institute epidemiologic experiments appears to be a type Aertrycke strain.⁴⁰ It was isolated in a spontaneous epidemic in cancer breeding stock.⁴¹ Another strain isolated from a different outbreak in the same stock was said to be "related but not identical with two strains of enteritidis."⁴² Both enteritidis and "animal paratyphoid" strains were also isolated in mouse epidemics by Topley, Weir and Wilson.⁴³

In rabbits Krumwiede, Valentine and Kohn have reported one strain (from Ferry) as of the "rodent type." Litch and Meyer⁴⁴ consider that the strain isolated by them from rabbits in a small laboratory outbreak belongs to this group. I have been able to procure only 3 strains of paratyphoid-enteritidis bacilli from rabbits. One is from the gallbladder of a rabbit in this laboratory that died after subcutaneous inoculation with a bacillus of the hemorrhagic septicemia group, and is *B. enteritidis*; one was received from the Lister Institute and is the strain isolated by McConkey⁴⁵ from cooked rabbit, consumption of which had apparently caused fatal illness in a child: this belongs to the Aertrycke type; the other was isolated by Dr. Lewis B. Bates, Ancon, Panama Canal Zone, from the spleen of a rabbit during a paratyphoid-like epidemic among rabbits and guinea-pigs, and is also of the Aertrycke type.

Porcine Strains.—The frequency with which paratyphoid-like bacilli are found in diseased swine has long been known; a member of this group, *B. suispestifer* (*B. cholerae suis*), was considered to be the cause

³⁹ Thomas: *Ibid.*, 1924, 34, p. 407.

⁴⁰ Webster, L. T.: *J. Exper. Med.*, 1922, 36, p. 97.

⁴¹ Lynch, C. J.: *Ibid.*, 1922, 36, p. 15.

⁴² Amoss, H. L., and Haselbauer, P. P.: *Ibid.*, 1922, 36, p. 107.

⁴³ *Jour. Hyg.*, 1921, 20, p. 227.

⁴⁴ *Jour. Infect. Dis.*, 1921, 28, p. 27.

⁴⁵ *Jour. Hyg.*, 1906, 6, p. 570.

of hog cholera⁴⁶ for some years before the discovery of a specific filtrable virus.

Whether bacilli of this group occur in healthy animals was early questioned, and although some of the first observers⁴⁷ answered affirmatively, the methods used were hardly adequate for definite identification. The majority of recent observers have failed to isolate from healthy swine any organisms that by appropriate cultural and serologic tests could be surely identified as belonging to this type. I have elsewhere⁴⁸ put on record the results obtained in the examination of 1,419 strains from the intestines of 291 normal swine, showing that no single culture was found to possess all the characteristic qualities. It may, I think, be reasonably concluded that organisms of the *B. suispestifer* type are not common inhabitants of the intestines of normal swine.

In natural outbreaks of hog cholera, "*B. suispestifer*" appears to be present so frequently that there is still doubt in the minds of some observers about its significance in this disease. In the course of routine serum production for prophylaxis against hog cholera, *B. suispestifer* may be isolated often from the blood of hogs inoculated with hog cholera virus.⁴⁹ *B. suispestifer* or a closely related type has also been isolated from animals dying from swine dysentery.⁵⁰ The majority of recent workers regard *B. suispestifer* not only as an important secondary invader, but also as the primary cause of certain outbreaks of disease in swine.

For a long time the opinion was widely current in Germany that *B. suispestifer* and *B. paratyphosus* B were identical, and the resultant confusion in the labeling of cultures has greatly retarded any clear differentiation. Many of the published descriptions justify only the statement that the bacillus dealt with belonged to the paratyphoid-enteritidis group. For these reasons, the study of porcine strains is beset with peculiar difficulties. Only those strains of known porcine origin can be properly considered for comparison. Mere labeling with the names "*B. suispestifer*" or "*B. aertrycke*" has to be practically disregarded.

⁴⁶ Many English writers have used the term *B. aertrycke* as strictly synonymous with *B. suispestifer*. The *B. aertrycke* was first isolated by de Nobele from an outbreak of food poisoning, and was apparently derived not from swine, but from a calf suffering from severe enteritis. There is reason to believe that *B. aertrycke* is different both from *B. suispestifer* and from the Schottmüller strain of *B. paratyphosus* B (Jordan: Jour. Infect. Dis., 1923, 33, p. 567).

⁴⁷ Uhlenhuth, Xylander, Hübener and Bohtz: Arb. a. d. k. Ges., 1907-1908, 27, p. 425. McConkey believed that contamination must have taken place in the consumer's house since the earlier meals from the incriminated rabbit meat had not caused illness. It seems possible, however, that the warming over process had, as in other instances, given opportunity for the multiplication of an initially small number of bacilli. Unequal distribution throughout the body of the rabbit may also have existed.

⁴⁸ Jordan: Jour. Infect. Dis., 1918, 22, p. 252.

⁴⁹ Doyle and Spray: Ibid., 1920, 27, p. 245.

⁵⁰ Smith and Reagh: Jour. Med. Res., 1903, 9, p. 270. Whiting, Doyle and Spray: Purdue Univ. Agr. Expt. Station, Bull., October, 1921, p. 257.

There is satisfactory evidence that at least 3 types of bacilli belonging to this group have been more or less frequently isolated from diseased swine: (1) *B. suispestifer*, (2) *B. paratyphosus* B (porcine) and (3) *B. typhi suis* or *B. suispestifer-voldagsen*. To these may be added (4) occasional findings of *B. enteritidis*.⁵¹

1. *B. suispestifer*.—As already pointed out,⁵² this type is characterized by certain cultural as well as serologic differences. Arabinose and dulcitol are attacked slowly or not at all, and lead acetate medium is usually not blackened.⁵³ In addition, it has been found that inositol is not fermented by any of the 38 strains of this series. On this point, my results are in agreement with those of Weiss and Rice.⁵⁴ Furthermore, none of these 38 strains ferment trehalose, thus confirming the results of Koser with 8 strains.⁵⁵ Serologically, a clear distinction exists between this cultural type and other members of the group. In an earlier paper,⁵² I have recorded the characteristics of 25 strains of known porcine origin; 20 of them were of the *B. suispestifer* type and 5 of the *paratyphosus* B type. Since then I have received, through the kindness of Professor R. E. Buchanan, 13 Iowa cultures: 1 from the kidney of a virus hog (*B. suispestifer* type), 1 stock "hog cholera" culture, exact history unknown (*paratyphosus* B. type) and 11 isolated in his laboratory from the feces of virus hogs (8 *B. suispestifer*, 2 *B. paratyphosus* B, 1 *B. enteritidis*). I have also received from Professor R. S. Spray, then of the veterinary department of Purdue University, 20 strains isolated directly from pigs inoculated with virus blood: 6 strains from the lung (all *B. suispestifer* type), 5 from the mesenteric lymph glands (4 *B. suispestifer*, 1 *B. paratyphosus* B type), 3 from the liver (all *B. suispestifer* type) and 6 from direct plating of virus (5 *B. suispestifer*, 1 *B. paratyphosus* B type). The Spray collection included in addition 6 strains isolated from rabbits (4 heart blood, 2 liver) that had received injections of virus blood; there is, consequently, some doubt as to whether these 6 strains were originally in the virus blood or were initially present in the rabbit body; 4 of these were of the *B. suispestifer* and 2 of the *B. paratyphosus* B type.

⁵¹ Tenbroeck: J. Exper. Med., 1918, 28, p. 759.

⁵² Jordan: Jour. Infect. Dis., 1917, 20, p. 457.

⁵³ Jordan and Victorson: Ibid., 1917, 21, p. 571. A few strains that in all other cultural characters resemble the *suispestifer* type blacken lead acetate medium. These are 279, 292, 297 (from rabbits inoculated with hog cholera virus blood), 353 (Lister Institute, food poisoning case) 359 (Lister Institute, mesenteric gland of monkey).

⁵⁴ Jour. Med. Res., 1917, 30, p. 403; Weiss: *ibid.*, 1917, 31, p. 135.

⁵⁵ Jour. Infect. Dis., 1921, 29, p. 67.

Fifty cultures have been studied, which were derived certainly or with a high degree of probability from swine; 38 of these were culturally and serologically of the *B. suipestifer* type, 10 of the *B. paratyphosus* B type, 1 of the *B. enteritidis* and 1 of the *B. voldagsen* type. The 2 series from Iowa and Indiana which came into my hands soon after their direct and undoubted isolation from swine numbered 32 cultures, 26 of which were of the *B. suipestifer* type. Although there are instances showing that *B. paratyphosus* B may be the more common or the sole type to be associated with certain hog cholera virus inoculations,⁵⁶ it seems probable, from these observations, that *B. suipestifer* is of more frequent occurrence in diseased swine and that the specific name is quite justified.

2. *B. paratyphosus* B.—These are organisms possessing all or most of the cultural characteristics at present recognized as distinguishing *B. paratyphosus* B isolated from human infections. They ferment xylose, arabinose, and dulcitol promptly, producing acid and gas within 24 hours. They also blacken strongly lead acetate medium and ferment trehalose and inositol.⁵⁷

It was pointed out in 1917⁵⁸ that some strains of unquestionably porcine origin gave the cultural reactions of the paratyphoid B. type and also stood closer to the latter than to *B. suipestifer* in agglutinative behavior. In later studies on the paratyphoid-enteritidis group,⁵⁹ Krumwiede, Kohn and Valentine⁶⁰ noted that "a few strains fall into neither group (*suipestifer* or paratyphoid B) according to their agglutinative reaction, although culturally they belong to one or the other of the above groups," but added doubtfully: "These last strains were received as of porcine origin." They concluded that "*B. paratyphosus* B is essentially a human pathogen," and that "infected swine are not a source of contagion for this disease, either directly or indirectly, through the consumption of infected food." Tenbroeck,⁶¹ however, isolated from pigs used in hog cholera experimentation 5 organisms which were culturally the same as paratyphoid bacilli isolated from man. Agglu-

⁵⁶ Tenbroeck: Jour. Exper. Med., 1918, 28, p. 759.

⁵⁷ Gas production in inositol medium is variable, and this probably accounts for the irregular results reported by some workers. I have found, however, that if the ability to attack this substance is measured by hydrogen-ion determination instead of by gas production, valuable information for differentiation is obtained. In the examination of several hundred organisms of the paratyphoid-enteritidis group, the *B. paratyphosus* B type is thus far the only one observed to attack inositol. A few otherwise typical *B. paratyphosus* B strains, however, give consistently negative results.

⁵⁸ Jordan: Jour. Infect. Dis., 1917, 20, p. 457.

⁵⁹ Ibid., 1918, 3, p. 89.

⁶⁰ Jour. Med. Res., 1918, 33, p. 89.

⁶¹ Jour. Exper. Med., 1918, 28, p. 759.

tinatively, they differed somewhat from the latter as well as from *B. suipestifer*.

It seems probable that much of the confusion that has centered in this group has been due to the occurrence in swine of at least 2 distinct types, and that the insistence by certain German writers on the identity of *B. suipestifer* and *B. paratyphosus* B owes its origin to the assumption that all "hog cholera bacilli" were alike, as well as to the application of insufficient differential tests. This confusion has been deepened by the identification of the "Aertrycke" strains with *B. suipestifer* by certain English bacteriologists. These so-called Aertrycke strains appear for the most part to have been isolated from human food poisoning cases and from outbreaks of disease in the guinea-pig and other animals. While *B. aertrycke* has been commonly regarded as strictly synonymous with *B. suipestifer* by most English writers, I have found that many of the strains labeled with this name correspond culturally with the *B. paratyphosus* B type of hog cholera bacillus. Some of the strains of German origin bearing the name *B. suipestifer* are certainly of the *paratyphosus* B. type. This was true of one received by me in 1914, which came from Kral's collection, and also of one sent to the Michigan Agricultural College by Ostertag (under the name *B. cholerae-suis*) and received by me from the latter laboratory.

Trawinski⁶² found cultural differences between strains isolated from "necessity slaughtered" swine and certain stock strains of *B. suipestifer* from various laboratory collections. Agglutinative differences were not observed, but the methods used by him were not suitable for bringing out differences of this character. Recent German workers are, however, recognizing the occurrence of 2 serologic types in swine.⁶³ Bruynoghe and Leynen⁶⁴ isolated a paratyphoid-like organism from swine dying from a respiratory infection. It did not resemble serologically various stock cultures of *B. suipestifer* with which it was compared. Fermentation tests with arabinose, etc., do not seem to have been made.

There are not many observations on the relative frequency of occurrence of the *B. paratyphosus* B and the *B. suipestifer* types in swine. Local differences doubtless occur. Tenbroeck (1918) in a study of 6 cultures from swine found 5 of the *paratyphosus* B type and 1 of the enteritidis type, but he did not find any of the *suipestifer* group. Three

⁶² Centralbl. f. Bakteriöl., I., O., 1917-18, 80, p. 339.

⁶³ Tormann, E.: Centralbl. f. Bakteriöl., I., O., 1919, 82, p. 532; Manteufel and Beger: Centralbl. f. Bakt., I. Orig., 1921-22, 87, p. 161.

⁶⁴ Ann. de l'Inst. Pasteur, 1921, 35, p. 261.

series of cultures with definite porcine history which I have worked out—2 from Iowa, 1 from Indiana—have yielded 36 strains of the *suipestifer* type; 8 of the *paratyphosus* B type and 1 of the *enteritidis* type. Six strains isolated from rabbits injected with hog cholera virus blood and hence perhaps of porcine origin were in 4 instances of the *suipestifer* type, and in 2 of the *paratyphosus* B type. Thirteen cultures, mostly laboratory stock, from various sources, but all of porcine origin certainly or with a high degree of probability were 10 of the *suipestifer* and 3 of the B type. There is thus ample evidence that on the continent of Europe and in several parts of the United States strains culturally resembling *B. paratyphosus* B occur more or less frequently in the bodies of swine affected with hog cholera, either naturally or in the course of experimentation.

In another paper,⁶⁵ it has been shown that 7 strains of undoubted porcine origin possess cultural and agglutinative characters similar to those of strains from human paratyphoid fever (*Schottmüller* type). Two strains from rabbits that had been inoculated with hog cholera virus were also of this type. The question may be raised whether these swine paratyphoids are in all respects similar to the *paratyphosus* B bacilli found in human paratyphoid fever. Tenbroeck⁶⁶ is inclined to answer this in the negative, but the strains with which he worked seemed to have belonged to the *Aertrycke* rather than to the *Schottmüller* type. The 7 strains of porcine origin in my collection, by the absorption test, show no differences from the human strains.

Tenbroeck found that his swine typhus strains when injected into rabbits produced immunity to *B. suipestifer*, while human paratyphoid strains did not. To test this point with my porcine strains, rabbits were immunized severally against 7 swine paratyphoid and 6 human paratyphoid strains. Each animal was given 9 successive subcutaneous and intraperitoneal injections as for the production of agglutinins. The agglutinin titer, at the end of this period, was in most cases about 1:5,000, in 2 instances as low as 1:1,000, in 2 as high as 1:10,000. The rabbits were then inoculated subcutaneously with one M L D of *B. suipestifer*. The control rabbit (2,320 gm.) died in 4 days. Five of the 6 rabbits immunized with human paratyphoid strains died (2 in 5, 2 in 6, 1 in 7 days). Three of the 7 rabbits immunized with porcine paratyphoid strains died (1 in 4, 1 in 5, 1 in 6 days). All the 5 rabbits

⁶⁵ Jordan: Jour. Infect. Dis., 1923, 33, p. 567.

⁶⁶ J. Exper. Med., 1918, 28, p. 759; 1920, 32, p. 19.

that survived showed loss of weight, diarrhea and fever. While the proportion of rabbits immunized with human paratyphoid strains which survived was less than that of the rabbits immunized with porcine strains, the difference is not large enough to be significant. It may be safely concluded that no considerable degree of immunity to *B. suipestifer* was produced by a rather thorough-going serial inoculation with porcine paratyphoid B strains.

3. *B. voldagsen*.—This organism which was isolated by Dammann and Stedefeder⁶⁷ from an outbreak of infectious disease in swine is usually considered identical or nearly so with *B. typhi-suis* (or *B. glässer*) isolated from a similar outbreak by Glässer about the same time. The relation of these strains to disease in young pigs (Ferkeltyphus, shoat typhoid) has been the subject of some dispute, but most writers regard them as the cause of shoat typhoid and as “variants” of *B. suipestifer*.⁶⁸ Manninger,⁶⁹ however, found true *B. suipestifer* and not *B. voldagsen* in shoat typhoid in Hungary. Manteufel, Zschucke and Beger⁷⁰ regard the Voldagsen bacillus as a variety of *B. suipestifer*, and Manninger notes that a “Glässer” strain from the Koch Institute collection behaves like *B. suipestifer*. Pfeiler and Engelhardt^{70a} consider the shoat typhoid bacillus an independent member of the colityphoid group. The descriptions of the Voldagsen type given by German writers show considerable variation, and it seems probable that not all the strains to which this designation has been applied were identical; according to Weidlich,⁷¹ some strains produce indol, others not. Tormann⁷² did not find any “Ferkeltyphus” bacilli (voldagsen?) in healthy swine. Bernhardt⁷³ has reported finding an organism resembling the Voldagsen-Glässer bacillus in necropsy material from a food poisoning case apparently due to the use of meat from a cow slaughtered through necessity. Neukirch⁷⁴ in observations on a disease in Turkey resembling dysentery isolated bacilli which agglutinated only in low dilution (1:360) with a highly potent *B. paratyphosus* B serum. Cultures were sent to the Koch Institute in Berlin

⁶⁷ Arch. f. wissenschaft. u. prakt. Tierheilk., 1910, 36, p. 432.

⁶⁸ Uhlenhuth and Haendel: Schweinepest und Schweineseuche, in Kolle und Wassermann's Handbuch, 1913, p. 397.

⁶⁹ Centralbl. f. Bakteriologie, I, O., 1922, 89, p. 23.

⁷⁰ Ibid., 1921, 86, p. 214.

^{70a} Ztschr. f. Immunität, I, O., 1919, 28, p. 434.

⁷¹ Berl. tierärztliche Wechschr., 1914, 30, p. 73.

⁷² Centralbl. f. Bakteriologie, I, O., 1919, 82, p. 532.

⁷³ Ztschr. f. Hyg., 1912-13, 73, p. 65.

⁷⁴ Berl. klin. Wechschr., 1917, 15.

where they were agglutinated in high dilution with the type Glässer-Voldagsen serum. Detailed agglutination-absorption tests and full cultural studies do not seem to have been made.

A culture bearing the name *B. voldagsen* was sent me by Dr. W. E. King of Detroit, who had received it from Dr. Haendel. A culture received from the same source at the same time under the name *B. typhi-suis*, Glässer, was culturally almost similar to *B. voldagsen* although agglutinatively not entirely identical.

The "*Voldagsen*" strain possesses the main cultural characteristics of the group. In fermentation reactions, it stands closer to the *B. paratyphosus* B type than to the *B. suipestifer*, since it ferments readily arabinose, trehalose, dulcitol; it does not, however, blacken lead acetate medium or ferment inositol. It does not agglutinate at 1:250 with high titer (1:5,000) serum for *B. typhosus*, *B. paratyphosus* A or *B. enteritidis*. It agglutinates 1:250 to 1:500 with *B. paratyphosus* B. (*Schottmüller* and *Aertrycke* varieties) and *B. suipestifer* serum. It agglutinates to the titer limit with *B. paratyphosus* C. serum.⁷⁵ By the absorption method, it was found that the *Voldagsen* strain removed the agglutinin for *B. paratyphosus* C from the homologous serum, but did not do this for the 2 specific varieties of the *B. paratyphosus* B serum or for the *B. suipestifer* serum. So far as conclusions can be drawn from cultural and agglutination tests, this particular strain labeled *Voldagsen* is more closely related to the *B. paratyphosus* C. type than to other members of the group. Its relation to other strains that have received the same designation is uncertain.⁷⁶

Bovine Strains.—Bacilli of the paratyphoid-enteritidis group have been reported as occurring in the intestines of healthy calves or cattle.⁷⁷ Most observers, however, have failed to find true paratyphoid-enteritidis bacilli in the intestines of healthy bovines.⁷⁸ My own observations in this direction have been made on the intestinal contents of 52 presumably normal calves killed in the Union Stockyards, Chicago, between Jan. 17 and March 21, 1918. The samples were obtained and examined in

⁷⁵ This serum was prepared with the Witts strain of *B. paratyphosus* C kindly sent me by Dr. F. W. Andrews.

⁷⁶ Similar results were obtained by Andrews and Neave: *Brit. J. Exp. Path.*, 1921, 2, p. 157.

⁷⁷ Uhlenhuth and Hübener: *Med. Klin.*, 1908, 48, p. 1823. Eckert: *Inaug. Diss.*, cited by Hübener: *Fleischvergiftungen und Paratyphusinfektionen*, 1910.

⁷⁸ Horn and Huber: *Centralbl. f. Bakteriologie*, I, O., 1911-12, 61, p. 452. Aumann: *Centralbl. f. Bakteriologie*, I, O., 1911, 57, p. 310. Titze and Weichel: *Arch. a. d. k. ges.*, 1909-10, 33, p. 516. Savage: *Medical Officer's Report, Local Gov't Bd.*, 1907, p. 253; 1908, p. 425. Christiansen: *Centralbl. f. Bakteriologie*, I, O., 1916-17, 79, p. 196. Fischer: *Centralbl. f. Bakteriologie*, I, O., 1915-16, 77, p. 6.

the same manner as those from swine described in an earlier paper.⁷⁹ The total number of colonies picked from Endo plates was 194. The majority of these (140) turned out to be definite varieties of *B. coli*. Fifty of the whole number of cultures failed to give gas in dextrose, and 24 liquefied gelatin. There is a close correlation between inability to liquefy gelatin and failure to produce gas in dextrose, 22 out of the 24 nonliquefiers belonging in this class. The 2 liquefying gas-producing strains were apparently of the proteus type. Not a single one of the colonies selected for examination gave paratyphoid-like characters, i. e., dextrose +, lactose —, and nonliquefaction of gelatin. So far as this series is concerned, the results of searching for organisms of the paratyphoid group in the intestines of normal calves were entirely negative.

While it thus seems unlikely that bacilli of this group occur commonly in the intestines of normal bovines, they have been found frequently in association with various diseases and pathologic conditions, notably epidemic diarrhea or dysentery of calves, as well as in outbreaks and isolated cases of pneumonia, septicemia and mastitis. They have also been found in necrotic areas in the liver and other organs, whence the name *B. nodulifaciens bovis* applied to some bovine cultures. The unfortunate name of paracolon bacillus was given by Jensen to bacilli found in calf dysentery. According to Jensen⁸⁰ "paracolon bacilli" may be grouped culturally into two types, distinguished by their ability to ferment arabinose, and serologically into three: one similar to *B. enteritidis*, another—a small number—to *B. paratyphosus* B and a third influenced in no significant degree by agglutinins for either group. In other instances, definite identification with known bacterial species is hardly possible from the original description.⁸¹ The majority of articles, however, that I have been able to consult, point to *B. enteritidis* as the organism of this group most commonly present in bovine infections.⁸² Another reason, possibly, for supposing *B. enteritidis* to be the most common member of this group in bovine infections is its relatively frequent isolation in meat poisoning outbreaks in which the incriminated food was derived from cattle or calves. Savage,⁸³ in a tabular summary of

⁷⁹ Jordan: Jour. Infect. Dis., 1918, 22, p. 252.

⁸⁰ Kolle and Wassermann: Handb. d. path. Mikr., Ed. 2, vol. 6, p. 135.

⁸¹ Thomassen: Ann. de l'Inst. Pasteur, 1897, 11, p. 523. Mohler and Buckley: 19th Annual Report of the Bureau of Animal Industry, 1902, p. 297. A culture of the Thomassen bacillus, however, was subsequently identified by Savage (Jour. Hyg., 1912, 12, p. 1) as *B. enteritidis*. Stickdon: Centralbl. f. Bakteriöl., 1915, I, O., 76, p. 245.

⁸² Pitt: Ibid., 1909, 49, p. 593. Meissner and Kohlstock: Ibid., 1912, 65, 139. Meyer, Traum and Roadhouse: Jour. Am. Vet. Med. Assn., 1916, 49, p. 17. Winzer: Ztschr. f. Fleisch u. Milchhyg., 1911-12, 22, p. 81. Riemer: Centralbl. f. Bakteriöl., I, O., 1908, 47, p. 169.

⁸³ Food Poisoning and Food Infection, Cambridge, England, 1920, pp. 76-80.

British food poisoning outbreaks between 1878 and 1918, lists 42 outbreaks in which there was more or less conclusive evidence that beef, veal or milk was the cause of the illness. On analyzing these, it appears that in 21, bacterial examination was lacking or was inconclusive, in 9 a group identification ("Gaertner") only was made, in 4 the organisms were identified as "*B. suis*pestifer" (all of these were before 1900!), while in 8, including all the recent cases, *B. enteritidis* was isolated.

Karsten⁸⁴ regards *B. enteritidis* as the common cause of calf paratyphoid.

There are also a number of instances in which an organism identified as *B. paratyphosus* B or closely related to the latter has been reported in cattle.⁸⁵ Zschiesche⁸⁶ found that while "paracolibazillosis" due to *B. enteritidis* made up the chief body of infections of calves in East Prussia, similar disease manifestations were occasionally evoked by other microbes, and that among the latter *B. paratyphosus* B or organisms of that type were more frequently present than sometimes assumed. Karsten's⁸⁴ observations correspond with his view.

Although, as shown in the foregoing, bacilli of the paratyphoid-enteritidis group have been found not infrequently in various bovine infections, cultures of bovine strains are not common in laboratory collections. Some cultures isolated from cattle and sent to me under various designations have proved to be lactose or saccharose fermenters or to depart in other fundamental ways from the type standard. In the course of several years of collecting, I have obtained only 3 cultures of true paratyphoid-enteritidis bacilli of definite bovine origin.⁸⁷

205. From stool of calf suffering from epidemic diarrhea. Typical *B. enteritidis*. Agrees with original description by K. F. Meyer: Jour. Infect. Dis., 1916, 19, p. 700.

- | | | |
|--|---|---|
| Inosit-negative
Paratyphoid B
Aertrycke type | { | 358. From blood of calf, epidemic diarrhea, Mesopotamia. Lister Institute, Collection of Type Cultures No. 32. |
| | { | 389. From general outbreak in heifers, involving small and large intestines. Received from Theobald Smith. Outbreak not reported. |

Ovine Strains.—Sheep are apparently less frequently affected than cattle or horses. Andrejew's observations⁸⁸ on the occurrence of "bac-

⁸⁴ Der Paratyphus der Kälber, Berlin, 1921, pp. 109.

⁸⁵ Schmitz: Ztschr. f. Fleisch. u. Milchhyg., 1914, 29, p. 145. Zwick and Weichel: Arb. a. d. k. Gsndhkamte, 1910, 34, p. 391. Ledschbor: Ztschr. f. Inf. d. Haustiere, 1909, 6, p. 380. Zeller: Ztschr. f. Inf. d. Haustiere, 1909, 5, p. 361.

⁸⁶ Centralbl. f. Bakteriologie, I, O., 1917-18, 80, p. 350.

⁸⁷ The original *B. aertrycke* strain was apparently of bovine origin.

⁸⁸ Arb. a. d. k. Ges., 1909-10, 33, p. 363.

teria of the hog cholera group" in the intestinal tract of sheep are open to the criticism that inadequate identification methods were used. It is significant that relatively few outbreaks of food poisoning have been attributed to mutton; Savage in his comprehensive summary of British experience⁸⁹ records only one instance in which the cause was "possibly mutton." The well-known "mutton" strain of the Lister Institute collection, however, seems to have been isolated from a case of food poisoning in man in Newcastle in 1911. This is a typical *B. paratyphosus* B organism of the Aertrycke type.⁹⁰ Frickinger,⁹¹ who isolated a bacillus of the *B. paratyphosus* B type from liver sausage and from the stools of sick persons in an extensive food poisoning epidemic in Germany (1,500 cases, 4 deaths), traced the infection to an outbreak of diarrheal disease in sheep; it had been supposed that the illness of the sheep was due to the use of a new salt lick, and 32 of the animals were slaughtered and the carcasses sold for food. Young and Dawson⁹² have reported an outbreak in Manchester apparently due to mutton, in which the bacillus isolated agreed in all respects with the type "mutton" strain. A culture of this organism kindly sent me by Dr. Dawson has proved, like the other mutton strain, to have all the features of the Aertrycke type.⁹³ Two strains of a bacillus isolated from an extensive outbreak of dysentery in lambs (30,000 were affected!) in Colorado in 1923 were sent to me by the kindness of Dr. Newsome and Dr. Geiger. The strains are identical, and like the 2 mutton strains discussed above, are typical inositol-fermenting *B. paratyphosus* B bacilli of the Aertrycke type. The same identification has been made by Newsome and Cross⁹⁴ in their report of the outbreak.

All 3 strains of ovine origin that I have secured are, therefore, *B. paratyphosus* B, Aertrycke type.

Miscellaneous.—Besides the group epidemics and definitely pathogenic manifestations in various mammals that have been described above, there are on record a number of more or less isolated instances in which bacilli of the paratyphoid-enteritidis group have been encountered in other mammalian species. As in the cases already discussed, uncertainty exists as to the precise nature of the findings. In many instances, the

⁸⁹ Food Poisoning and Food Infections, Cambridge, 1920.

⁹⁰ Jordan, E. O.: Jour. Infect. Dis., 1923, 33, p. 567.

⁹¹ Ztschr. f. Fleisch u. Milchhyg., 1919, 29, p. 346.

⁹² Lancet, 1922, 2, p. 608.

⁹³ Jordan, E. O.: Jour. Infect. Dis., 1923, 33, p. 567.

⁹⁴ Jour. Am. Vet. Med. Assn., 1924, 66, p. 289.

only statement warranted by the description given is that the micro-organism found apparently belonged to the paratyphoid-enteritidis group.

In canine distemper Torrey and Rahe⁹⁵ isolated a bacillus identified by them as *B. enteritidis* (Gärtner), which they considered a secondary invader.

Mori⁹⁶ reported finding a bacillus of this group in several cats, but his description does not permit identification. A strain from a cat was included by Krumwiede, Valentine and Kohn⁹⁷ in their study of rodent strains. It apparently belonged to the Aertrycke type. Two cultures in my collection, both from the Lister Institute, are from unusual animal sources. No. 359, Lister Institute no. 91-G, is from the mesenteric gland of a monkey that died in the course of a dietetic experiment. This I have found to be a typical *B. suispestifer* with the definite cultural and agglutinative character of this type.⁹⁸ No. 361 is from the spleen of a skunk dying in an epizootic on a skunk farm in the north of England. This is a *B. paratyphosus* B of the Aertrycke type.

SUMMARY AND CONCLUSION

A review of the existing data and a study of more than a hundred strains of paratyphoid-enteritidis bacilli from various mammalian sources (other than human) have shown that certain peculiarities of distribution and immunologic relationship characterize the various members of the group.

B. paratyphosus A is apparently an exclusively human strain; no strain in my collection comes from other than human sources. Neither have I found any authentic instance recorded elsewhere of natural animal infection with this type.

In the horse, a specialized member of the group, *B. abortivo-equinus*, predominates and is perhaps the only organism of the group producing natural infection in equines. This bacillus has evidently become rather closely adapted to its equine host, since it possesses special cultural and agglutinative features, and since it seemingly has never been found in infections of other animals.

In rodents (chiefly mice, rats, guinea-pigs and rabbits), two types are found: *B. enteritidis* is the type most commonly reported in infections of laboratory rats, and the commercial rat virus chiefly contains this

⁹⁵ Jour. Med. Res., 1912-13, 27, p. 291.

⁹⁶ Centralbl. f. Bakteriöl., I. O., 1905, 38, p. 42.

⁹⁷ Jour. Med. Res., 1919, 39, p. 449.

⁹⁸ Jour. Infect. Dis., 1917, 20, p. 457.

organism. Mice are sometimes infected with this organism, but the proportion of enteritidis and Aertrycke infection is not known. In laboratory animals (white mice, white rats, rabbits, guinea-pigs) reported outbreaks may not give a correct idea of relative natural susceptibility, since any particular infection may be widely disseminated, owing to the shipment of infected stocks to different parts of the country. Healthy carriers are probably numerous. Several observers have found both enteritidis and Aertrycke types in one and the same epidemic among laboratory animals. The majority of the guinea-pig strains in my collection are of the Aertrycke type (10 Aertrycke, 6 enteritidis). Both types also occur in infections in rabbits.

In the pig, as in the horse, definite host specialization has occurred. Of 50 cultures certainly from swine, 38 belong culturally and serologically to the *B. suis* type. I have not encountered this type of organism among strains from rodent or bovine sources, and the only other strain of animal origin in my collection which belongs to this type is from the monkey. Besides the characteristic porcine type, however, other members of the group occur in swine. Of the 50 strains from swine examined, 10 are of the *B. paratyphosus* B group, all type Schottmüller. Other investigators have perhaps had type Aertrycke strains from the pig, but none of them has come into my hands. Two other types, *B. enteritidis* and *B. veldagsen* ("*B. paratyphosus* C"?) have also been found in swine.

Bovine strains, so far as available for study and so far as can be gathered from other reports, are usually either *B. enteritidis* or *B. paratyphosus* B, Aertrycke type. The few sheep strains studied (3) are all *B. paratyphosus* B, Aertrycke type.

It is evident that host specialization has occurred to a considerable extent in man (*B. paratyphosus* A), the horse (*B. abortivo-equinus*) and the pig (*B. suis*). Less definite or less advanced is the relationship of *B. paratyphosus* B, Aertrycke type to rodents; this organism is found not only in rodent diseases, but also in bovine and ovine infections; it may possibly also sometimes occur in swine. In human paratyphoid fever, *B. paratyphosus* B, Schottmüller type, has been reported almost if not quite as frequently as *B. paratyphosus* A, but unlike the latter it has been found also in swine. The most cosmopolitan member of the group is apparently *B. enteritidis*, which is found not infrequently in human, porcine, bovine and rodent infections.

THEORY OF ELECTRICAL CONDUCTANCE OF INHOMOGENEOUS SYSTEMS WITH APPLI- CATIONS TO SUSPENSIONS OF BLOOD CELLS

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In a previous paper,¹ the authors proposed an approximate formula for the resistance of inhomogeneous bodies and applied it to suspensions of bacteria and yeast cells. In the meantime, a more accurate expression has been published.² We propose to discuss more rigorously the theory of the resistance of suspensions and to apply our new formula to experimental data.

THEORY OF ELECTRICAL CONDUCTANCE OF INHOMOGENEOUS BODIES

If a steady current is flowing in a system, the potential v at any point at which the conductivity is k , is a solution of the differential

$$\text{equation }^3 \quad \frac{\partial}{\partial x} \left[K \frac{\partial v}{\partial x} \right] + \frac{\partial}{\partial y} \left[K \frac{\partial v}{\partial y} \right] + \frac{\partial}{\partial z} \left[K \frac{\partial v}{\partial z} \right] = 0$$

This equation is of the same form as the one satisfied by the potential in an electrostatic field provided the dielectric³ constant at every point is proportional to K . Moreover, at the boundaries separating one kind of conductor from another, the conditions to be satisfied in the current problem are that both $K \frac{\partial v}{\partial n}$ and $\frac{\partial v}{\partial S}$ must be continuous, where $\frac{\partial}{\partial n}$ denotes differentiation along the normal to the boundary, and $\frac{\partial}{\partial S}$ denotes differentiation along any line in the boundary. Once more, these are the conditions to be satisfied in an electrostatic problem at the boundary between two dielectrics whose dielectric constants are equal to the two values of K . "Thus the equipotentials in this electrostatic problem coincide with the equipotentials in the actual current problem and the lines of force in the electrostatic problem correspond with the lines of flow in the current problem."⁴

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¹ Jour. Infect. Dis., 1924, 34, p. 192.

² MacDougall, F. H.: Science, 1924, 59, p. 403.

³ Jeans: Mathematical Theory of Electricity and Magnetism, 1920, p. 345.

⁴ Ibid., p. 346.

ELECTROSTATIC PROBLEM

Let us consider first a single sphere of radius R in an infinite medium, the dielectric constant of which is unity. Let the dielectric constant of the sphere be K_2 . If the original uniform field is given by the equation

$$(2) \quad V = -FX$$

the expression for the potential at any point outside the sphere is found to be

$$(3) \quad V = -FX + \frac{K_2 - 1}{K_2 + 2} F \frac{R^3 x}{r^3}$$

where r is the distance of the point from the center of the sphere.⁵ According to equation (3), the sphere is equivalent to an electric doublet

whose moment, μ , is $\frac{K_2 - 1}{K_2 + 2} R^3 F$. In this case ⁶ we have

$$(4) \quad \mu = \frac{K_2 - 1}{K_2 + 2} R^3 F$$

Let us now suppose that throughout the medium of dielectric constant unity there are spheres of radius R and dielectric constant K_2 chaotically distributed at the rate of n per c.c. On account of the chaotic distribution of the spheres, a region which contains a large number of them may be treated as equivalent to a homogeneous body whose dielectric constant is K and in which the field may be considered as uniform and given by the equation

$$(5) \quad V = -\bar{F}X$$

Here \bar{F} is the mean electric intensity and K may be called the average dielectric constant. Fixing our attention on a single sphere, we find that its polarization will depend not only on the value of \bar{F} , but also on the polarization of the spheres in the neighborhood. The effective intensity E producing polarization of the spheres is given by the equation

$$(6) \quad E = \bar{F} + \frac{4}{3} \Pi P$$

In this equation, P stands for the polarization. We use the word in the sense employed by Richardson, but in equation (6) we express our results in ordinary electrostatic units. According to equation (4), we obtain the following expression for the moment, μ of each sphere considered as a doublet:

$$(7) \quad \mu = \frac{K_2 - 1}{K_2 + 2} R^3 E = \frac{K_2 - 1}{K_2 + 2} R^3 \left(\bar{F} + \frac{4}{3} \Pi P \right)$$

⁵ Ibid., p. 229.

⁶ Ibid., p. 51.

Since the polarization P is equal to the sum of the moments of the equivalent doublets in unit volume,⁷ we obtain

$$(8) \quad P = n \mu = \frac{K_2 - 1}{K_2 + 2} n R^3 E = \frac{K_2 - 1}{K_2 + 2} n R^3 \left(\bar{F} + \frac{4}{3} \Pi P \right)$$

From equation (8) we find

$$(9) \quad P = \frac{\frac{K_2 - 1}{K_2 + 2} n R^3 \bar{F}}{1 - \frac{K_2 - 1}{K_2 + 2} \frac{4}{3} \Pi R^3}$$

As defined by Richardson, the induction D , the polarization P , and the intensity F of a medium are related as follows:

$$(10) \quad D = \frac{K}{4 \Pi} \bar{F}; \quad P = \frac{K - 1}{4 \Pi} \bar{F}$$

Comparing equations (9) and (10), we obtain

$$(11) \quad K - 1 = \frac{\frac{K_2 - 1}{K_2 + 2} \frac{4}{3} \Pi n R^3}{1 - \frac{K_2 - 1}{K_2 + 2} \frac{4}{3} \Pi n R^3}$$

If we represent the term $\frac{4}{3} \Pi n R^3$ by a , we obtain

$$(12) \quad K = \frac{1 + \frac{2(K_2 - 1)a}{K_2 + 2}}{1 - \frac{(K_2 - 1)a}{(K_2 + 2)}}$$

in which a is the fraction of the volume occupied by the spheres.

We have supposed the medium in which the spheres are immersed to have a dielectric constant equal to unity. Evidently if we have spheres of dielectric constant K_2 in a medium of dielectric constant K_1 , we must replace K and K_2 of equation (12) by $\frac{K}{K_1}$ and $\frac{K_2}{K_1}$. In this general case, the average dielectric constant K of the system is given by the equation

$$(13) \quad \frac{K}{K_1} = \frac{1 + 2a \cdot \frac{K_2 - K_1}{K_2 + 2 K_1}}{1 - a \cdot \frac{K_2 - K_1}{K_2 + 2 K_1}}$$

From the equation, $D = \frac{K}{4 \Pi} \bar{F}$, we see that for a given potential gradient, the induction is proportional to K , the average dielectric constant.

⁷ Richardson: *Electron Theory of Matter*, 1914, p. 73.

CURRENT PROBLEM

From what was said in our introductory remarks, we infer that the solution we have obtained in equation (13) may be applied to determining the average conductivity K of a medium of conductivity K_1 , in which are immersed a large number of spheres of conductivity K_2 . In order to make equation (13) more directly applicable to experimental work, let us suppose that C is the "constant" of the cell in which the resistances are measured; s , the specific resistance of the suspended spheres; M , the resistance when the cell is filled with the menstruum or homogeneous medium; and R , the resistance when the cell is filled with the suspension. Let $S = Cs$. Then $C = R K = M K_1 = S K_2$, and equation (13) becomes

$$(14) \quad R = M \left[\frac{1 + a \left\{ \frac{S - M}{2S + M} \right\}}{1 - 2a \left\{ \frac{S - M}{2S + M} \right\}} \right]$$

We also obtain from (14)

$$(15) \quad a = \frac{(R - M)(2S + M)}{(2R + M)(S - M)} = \frac{2(R - M)}{2R + M} \cdot \frac{1 + \frac{M}{2S}}{1 - \frac{M}{S}}$$

In case the suspended particles have an infinite resistivity,

$$(16) \quad R = M \frac{1 + \frac{a}{2}}{1 - a} \quad \text{or} \quad a = \frac{2(R - M)}{2R + M}$$

Since the publication of these equations in *Science*, our attention has been called to two articles dealing with this subject in which equations are developed which are somewhat similar to ours. In the first place, Fricke⁸ obtains a result which may be written

$$\frac{K}{K_1} = 1 + 3a \frac{K_2 - K_1}{K_2 + 2K_1}$$

Fricke states:⁹ "This formula . . . will hold for a suspension so diluted that each sphere deforms the current lines of the original current independently of every other." In terms of our discussion of the electrostatic problem, Fricke assumes that the polarization of a given sphere is not affected directly by the polarization of the spheres in the neighborhood. In other words, this assumption is equivalent to identifying E and \bar{F} in equations (6) and (8). If this is done, equation (11)

⁸ Jour. Gen. Physiol., 1924, 6, p. 375.

⁹ Ibid., p. 382.

when suitably modified becomes identical with the equation proposed by Fricke. Moreover, if in our equation (13) we suppose that a is extremely small, we find that the equation proposed by Fricke is the limiting form. This is as it should be, for if a is very small, the spheres will be so far apart from each other that each may be treated as independent.

Dr. L. W. McKeehan of the Western Electric Company, New York, has kindly directed our attention to a paper by H. C. Burger¹⁰ who obtained 5 years ago a formula which is exactly the same as the one proposed by Fricke. Burger's formula is also explicitly deduced on the assumption that in the mixture of the two materials the volume concentration of one of them is very small.

METHOD OF TESTING THE EQUATIONS

If a series of measurements is carried out in which the volume concentration of the suspended material is varied while the specific resistance of the menstruum and that of the suspended material remain unchanged, and if R_1, R_2, R_3 , etc., represent the resistances of the suspension and a_1, a_2, a_3 , etc., are the corresponding values of a , then we find from equation (15),

$$(17) \quad a_1 : a_2 : a_3 : \dots :: \frac{2(R_1 - M)}{2R_1 + M} : \frac{2(R_2 - M)}{2R_2 + M} : \frac{2(R_3 - M)}{2R_3 + M} :$$

In other words, the expression $\frac{2(R - M)}{2R + M}$ is directly proportional to a .

Using a rectangular coordinate system, with values of $\frac{2(R - M)}{2R + M}$ as ordinates and values of a (or any quantity proportional to a) as abscissae, we should obtain a straight line passing through the origin. On the other hand, if the formula of Burger or Fricke is valid, we should have

$$(18) \quad a_1 : a_2 : a_3 : \dots :: \frac{2(R_1 - M)}{3R_1} : \frac{2(R_2 - M)}{3R_2} : \frac{2(R_3 - M)}{3R_3} :$$

In other words, the function $\frac{2(R - M)}{3R}$ should be directly proportional to the volume concentration of the suspended particles.

Altogether, the method just described furnishes an excellent test of the validity of equation (15); it does not permit of a determination of the values of S and a . These may be determined in the following way from a series of measurements of sufficiently high accuracy. In this series, the volume concentration of suspended material is kept constant

¹⁰ Physikal. Ztschr., 1919, 20, p. 73.

while the resistances of the menstrua are varied. In this case, let us suppose that R_1 , M_1 , R_2 , M_2 are the values of the resistance in two experiments in which a and S are supposed to be unchanged. If we represent the functions $\frac{2(R_1 - M_1)}{2R_1 + M_1}$ and $\frac{2(R_2 - M_2)}{2R_2 + M_2}$ by a_1 and a_2 , we obtain from equation (15),

$$(19) \quad \frac{1 + \frac{M_2}{2S}}{1 - \frac{M_2}{S}} = \frac{a_1}{a_2} \cdot \frac{1 + \frac{M_1}{2S}}{1 - \frac{M_1}{S}}$$

This is a quadratic equation in S from which the value of S is easily obtained. On substituting its value in equation (15), the value of a is arrived at. It should be observed that slight errors in R and M will cause large errors in the calculation of S , but will not affect the value of a to any great extent. It is, of course, possible to make use of all the data in a given series in calculating S and a , but the procedure is then much more tedious and involved.

TESTING OF FORMULA WITH HUMAN BLOOD CELLS

Fifteen c.c. of blood were obtained from 2 dispensary patients whose blood was apparently normal. The fibrin was removed by gentle stirring. The cells were removed by centrifuging and were then washed twice with 0.9% NaCl ($P_H = 7$), and the washings discarded. The cells were then suspended in 60 c.c. of NaCl and the suspension centrifuged. The supernatant liquid was removed and was used as the menstruum suspending the cells in the experiment. The liquid was removed as completely as possible from the packed cells which were thoroughly mixed and added in measured quantities to the menstruum in making up the suspensions used in the experiment. The resistance of the menstruum was measured before adding blood cells; and after the resistance of any suspension was measured, the blood cells were removed by centrifuging and the resistance of the menstruum again measured as a check. Suspensions were made up as follows:

TABLE 1
HUMAN BLOOD CELLS. 0.9% NaCl.

R	M	b	$\frac{2(R - M)}{2R + M}$	Ratio	$\frac{2(R - M)}{3R}$	Ratio
474.5	421.9	0.0909	0.0768	0.845	0.0739	0.813
516.7	421.9	0.1597	0.1303	0.816	0.1223	0.766
617.5	421.9	0.2857	0.2361	0.826	0.2112	0.739
711.5	421.9	0.3750	0.2139	0.837	0.2714	0.724

To 5 c.c. portions of 0.9% NaCl solution were added 0.5 c.c., 0.95 c.c., 2 c.c., and 3 c.c. of centrifuged and packed human blood cells. The values of a in this experiment were evidently proportional $\frac{0.5}{5.5}$, $\frac{0.95}{5.95}$, $\frac{2}{7}$ and $\frac{3}{8}$ or 0.0909, 0.1597, 0.2857 and 0.375. As has been shown above, if our formula is correct, the function $\frac{2(R-M)}{2R+M}$ should be proportional to these values, whereas if Burger's or Fricke's is correct, the function $\frac{2(R-M)}{3R}$ should be used. Let us represent the values 0.0909, 0.1597, etc., by the symbol b . In table 1 are given the values of R , M , b and the two functions just mentioned.

In column 5 of table 1 are given the values of the ratio of $\frac{2(R-M)}{2R+M}$ to b and in column 7, value of the ratio of $\frac{2(R-M)}{3R}$ to b . In the first case, the ratios are approximately constant, whereas in the second case, the ratios show a steady decrease. Our formula is represented very well by a straight line, whereas that of Burger or Fricke shows a decided departure from a straight line in the more concentrated suspensions.

DETERMINATION OF THE SPECIFIC RESISTANCE OF BLOOD CELLS AND THE VOLUME OCCUPIED

Blood cells were obtained from normal individuals and the serum removed by centrifuging. The centrifuged cells were mixed and then divided into 4 exact parts. Each part was washed twice with a salt solution, each part being treated exactly as any other except for the composition of the washing liquid. The 4 solutions used in washing contained 0.9%, 0.7%, 0.4%, and 0.1% NaCl and sufficient cane sugar was added to the last three to give the same ΔT as for the 0.9% NaCl. A series of suspensions was then made up in each of the foregoing solutions by adding those blood cells which had already been washed by the solution in each case. Four suspensions were made up with each solution, each of the suspensions having a volume of 10 c.c. and containing 1, 2, 3, and 4 c.c., respectively, of the packed red cells, the amounts being accurately measured with a pipet.

We may speak of volume concentrations of 10, 20, 30 and 40%, but the actual volume concentrations, although proportional to these numbers, will be somewhat smaller. We may call 10, 20, 30 and 40 the

apparent volume concentrations, confining the use of the symbol a to the actual values. The conductivity cell used in these experiments had a "cell constant" of 5.05, determined by means of KCl at 25°. The following tables give the results of the measurements and calculations.

TABLE 2
RESULTS OF MEASUREMENT AND CALCULATIONS WITH 0.9% NaCl

b	R	M	$\frac{2(R-M)}{2R+M}$	Ratio of $\frac{2(R-M)}{2R+M}$ to b
0.10	371.5	324	0.0890	0.890
0.20
0.30	512.6	324	0.0932	0.932
0.40	607.8	324	0.0922	0.922

TABLE 3
RESULTS OF MEASUREMENT AND CALCULATIONS WITH 0.7% NaCl

b	R	M	$\frac{2(R-M)}{2R+M}$	Ratio of $\frac{2(R-M)}{2R+M}$ to b
0.10	489.4	427.8	0.0876	0.876
0.20	573.6	427.8	0.1851	0.926
0.30	675.6	427.8	0.2786	0.929
0.40	784.7	427.8	0.3574	0.894

TABLE 4
RESULTS OF MEASUREMENT AND CALCULATIONS WITH 0.4% NaCl

b	R	M	$\frac{2(R-M)}{2R+M}$	Ratio of $\frac{2(R-M)}{2R+M}$ to b
0.10	880.4	775.4	0.0828	0.828
0.20	1007.0	775.4	0.1661	0.831
0.30	1174.0	775.4	0.2551	0.852
0.40	1347.0	775.4	0.3295	0.824

TABLE 5
RESULTS OF MEASUREMENT AND CALCULATIONS WITH 0.1% NaCl

b	R	M	$\frac{2(R-M)}{2R+M}$	Ratio of $\frac{2(R-M)}{2R+M}$ to b
0.10	3071	2746	(0.0731)	(0.731)
0.20	3329	2746	0.1240	0.620
0.30	3662	2746	0.1819	0.606
0.40	4084	2746	0.2452	0.613

In attempting to estimate the specific resistance of the blood cells from the data in tables 2, 3, 4 and 5, we have taken an average of the

values given in the 5th columns, giving the greatest weight to the results obtained for the largest value of b . Thus we adopted the following mean values: for the 0.9% solution 0.922; for the 0.7% solution 0.916; for the 0.4% solution 0.834, and for the 0.1%, 0.613. Equation (15) becomes in these cases

$$\begin{aligned}
 (20) \quad a &= n \times 0.927 \frac{1 + \frac{M_1}{2S}}{1 - \frac{M_1}{S}} = n \times 0.916 \frac{1 + \frac{M_2}{2S}}{1 - \frac{M_2}{S}} \\
 &= n \times 0.834 \frac{1 + \frac{M_3}{2S}}{1 - \frac{M_3}{S}} = n \times 0.613 \frac{1 + \frac{M_4}{2S}}{1 - \frac{M_4}{S}}
 \end{aligned}$$

where $M_1 = 324$, $M_2 = 427.8$, $M_3 = 775.4$, $M_4 = 2746$, and n has the series of values 0.1, 0.2, 0.3 and 0.4.

$$\text{From the equation } 0.927 \frac{1 + \frac{324}{2S}}{1 - \frac{324}{S}} = 0.613 \frac{1 + \frac{2746}{2S}}{1 - \frac{2746}{S}} \text{ in equations}$$

(20) we obtain $S = 9,700$. Using this value of S we obtain the 4 values of a .

$$a = 0.975 \, n$$

$$a = 0.979 \, n$$

$$a = 0.942 \, n$$

$$a = 0.975 \, n$$

Since the cell constant was 5.05, we obtain the following value for the specific resistant of the cells; viz., $s = \frac{S}{5.05} = 1925$ ohms. On account of the experimental error, this value of the resistance may be in error by 20 or 30%.

The data we have obtained in these experiments have been discussed at some length in order that we may make clear the method we have adopted in testing our equations and in applying them to the determination of the specific resistance of bodies suspended in a medium of different conductivity. Further investigations are under way with the object of increasing the experimental material and obtaining additional information in regard to the electrical resistance of suspensions.

SUMMARY

A rigorous development is given for the following equation giving the resistance of spherical conductors suspended in a conducting medium.

$$R = M \cdot \frac{1 + a \left(\frac{S - M}{2S + M} \right)}{1 - 2a \left(\frac{S - M}{2S + M} \right)}$$

Where M = resistance of suspending medium.
 a = volume occupied by conductors.
 S = resistance of cells.
 R = resistance of suspension.

Methods for checking the accuracy of this equation and other equations are described.

This equation is found to hold for very concentrated suspensions while other equations tested do not hold under these conditions.

The specific resistance of normal human red blood cells is found to be about 2,000 ohms.¹¹

¹¹ A result has been obtained by Maxwell and published in his treatise, "Electricity and Magnetism," which is apparently identical with our formula. Our method of deduction is different from that used by Maxwell and our deduction indicates a greater range of validity for our formula than was claimed by Maxwell.

"HORMONE" MEDIAUMS

SIMPLE METHOD OF PREPARATION AND VALUE OF HORMONE BLOOD AGAR FOR PRESERVING PNEUMOCOCCI AND STREPTOCOCCI

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The report of "hormone" mediums by Huntoon¹ a few years ago has brought about a complete revolution in the preparation of bacterial mediums. Huntoon gave credit to Lloyd² and to Cole and Lloyd³ for the principles on which he developed his medium, and he designed his technic (*a*) to extract the growth factors or "hormones" by bringing colloidal solutions into contact with meat and blood; (*b*) to preserve these factors by reducing the amount of heating, and by eliminating the filtration through substances which adsorb them (such as cloth, paper, fiber, etc.); (*c*) to supply sufficient amino-acids by the use of a suitable peptone and by the addition of egg yolk, and (*d*) to keep the hydrogen-ion concentration in a proper zone.

Shortly after Huntoon's report, we began experimenting with "hormone" mediums, and recently we have devised a modification of his method which we believe has certain advantages. The newer method permits the retention of all of the essential qualities of other "hormone" mediums and at the same time insures a less expensive and clearer medium than that obtained by following Huntoon's original directions. Further, we believe that we actually increase the "hormone" content by bringing the uncooked meat into contact with the melted agar before any coagulation of the former has occurred. It will be noted that we have omitted the egg. We admit that it aids somewhat in the filtration, but it is not actually necessary for that purpose, and experience has shown that it is not essential as a source of amino acids. Some work now in progress indicates that the addition of egg is often a source of stubborn contamination. Our method has proved so reasonable in cost and so simple in preparation that we use it for all routine mediums as well as for the class work.

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¹ Jour. Infect. Dis., 1918, 23, p. 169.

² Jour. Path. & Bacteriol., 1916, 21, p. 113.

³ Ibid., 1917, 21, p. 267.

MODIFIED "HORMONE" AGAR

1. Dissolve in 1 liter of distilled water 15 gm. of agar-agar shreds which have previously been washed thoroughly in running water and allow to cool to between 50 C. and 60 C. This step we believe to be important, because it reduces the amount of heating necessary after the addition of the meat and brings the agar suspension into contact with all of the meat particles before any coagulation has occurred.

2. Add 500 gm. of lean beef or beef heart (chopped to moderate fineness), bring to a boil and cook slowly for 15 or 20 minutes.

3. Filter through an ordinary round flour sieve (cullender type) about 16 mesh to the inch. Allow the agar and meat mixture retained on the sieve to become more or less evenly spread out, so that it acts as a filter, and again pour the filtrate through it. Filtration carried on in this way occurs with sufficient rapidity to allow several filtrations before cooling, if such are necessary to make the medium entirely clear. Egg need not be used.

4. Add peptone, 10 gm., and sodium chloride, 5 gm. Boil for about 5 minutes.

5. Correct to the desired reaction (P_H 7.5).

The sodium hydroxide used to correct the reaction is likely to cause a brownish discoloration of the medium if it is added before the meat is removed by filtration. This does not occur when it is added after the meat has been filtered out.

6. Allow to stand in a warm place for a few minutes until the precipitate produced by the hydroxide settles out and then decant the supernatant fluid.

With this procedure little precipitate is formed and little waste occurs.

7. Tube and sterilize by the fractional method or autoclave for about 20 minutes at 5 lbs. of pressure.

We have found that the medium is not damaged by autoclaving at low pressure, and we are now making a practice of autoclaving tubed medium once and the medium in flasks twice.

MODIFIED "HORMONE" GELATIN BROTH

Select a good gelatin, weigh out 10 gm. and dissolve it in a liter of distilled water, then proceed exactly as in the method described above.

The length of time over which the usually short-lived cultures of streptococci and pneumococci may be preserved on modified "hormone" blood agar seems worthy of note. The hormone agar was prepared, according to the method described, from equal parts of chopped lean beef and of beef heart, and about 5 parts per hundred of fresh, defibrinated human blood were added. The cultures were obtained from various sources and plated on hormone blood-agar plates. The various strains were identified in hormone sugar broths and transferred to the hormone blood-agar slants and incubated over night at 37 C. The tubes were then sealed with paraffin and placed on a shelf at room temperature near a window and without light protection. Every 3 months all of the cultures were transplanted and the tubes were again sealed and returned to the shelf.

Out of 68 cultures of pneumococcus, all grew on transplantation at the end of 3 and 6 months; 65 grew at the end of 9 months, and 62 were still living at the end of a year.

Of the 27 cultures of *Streptococcus pyogenes*, and 5 cultures of *Streptococcus viridans*, all were alive when transplanted at the end of the twelfth month.

SUMMARY

The method of making "hormone" medium which has been described produces a medium which is rich in growth-producing factors because the agar has already been melted and cooled when brought into contact with the raw uncoagulated meat particles; which retains these factors because they are not removed by the manner of filtration or by overheating; which is economical because the meat is removed by filtration so that the relatively large amount of medium usually cut off and thrown away is saved, and which is clear and free from discoloration because the reaction is not adjusted until after the meat has been removed.

Sixty-two cultures of pneumococcus and 32 cultures of streptococcus were kept alive for 12 months without transplantation, on modified hormone blood agar slants in tubes sealed with paraffin kept in the light and at room temperature.

MICROBIC RESPIRATION

IV. THE SO-CALLED AEROBIC GROWTH OF ANAEROBES: POTATO RESPIRATION *

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SYNOPSIS

Introduction

Microbic Association; Reducing Action of Chemicals; Animal Tissues;
Plant Tissues.

Potato Respiration

Methods

Raw Potato in Air; Effect of Autoclaving

Respiratory Quotient

Respiration in Pure Oxygen; in Pure Nitrogen; Alcohol Production

Respiration of Immersed Potato

The Potato and Growth of Anaerobes

Broth Culture with Distant Potato; Plate Culture

Microbic Association

Broth Culture with Distant *B. subtilis*; Plate Culture

Mixed Cultures with Pneumococcus

Discussion

Summary

INTRODUCTION

The prime characteristic of anaerobic organisms is their growth in the absence of air, and more especially of oxygen. Deserving of emphasis is the fact that the amount of oxygen which inhibits their growth is very small. This is demonstrated whenever these organisms are inoculated into mediums such as broth, gelatine or agar. Although the overhead air contains 20.9% of oxygen, the organisms are really exposed only to the dissolved oxygen, the actual amount of which, though clearly quite low, is nevertheless sufficient to prevent multiplication.

According to Matzuschita,¹ 5 different anaerobes produced spores in agar or gelatine mediums, under an exhausted bell-jar containing 0.0003% of oxygen, but failed to grow in a partial vacuum of 12.4 mm. pressure, representing 0.34% of oxygen. From the latter atmosphere, provided the pressure was normal, 10 c c. of water, at 0 degree and 760 mm., would dissolve 0.00136 c c. of oxygen, or 0.0136 volume per cent. This amount and even less, since the cultures were made at room temperature, is therefore inhibitive. On the other hand, Chudiakow² was able to grow anaerobes at 5, 10, 20 and 40 mm. of air pressure, the limit varying with the species. These values, representing

* Received for publication, Nov. 1, 1924.

¹ Arch. f. Hyg., 1902, 43, pp. 319-359.

² Centralbl. f. Bakteriöl., II, 1898, 4, pp. 389-394.

0.15 to 1.2% oxygen, recalculated for dissolved oxygen on the assumption of normal pressure, would give an approximate threshold range of from 0.006 to 0.048 volume per cent.

From a full atmosphere of oxygen, at 0 degree and 760 mm. pressure, pure water will dissolve 4.114 volume per cent. of the gas. It follows that from ordinary air, in which the partial pressure of oxygen is 20.9%, pure water under the foregoing conditions of temperature and pressure would take up 0.86 volume per cent. At 20 degrees, it would take up 0.59 volume per cent. of oxygen; and at 37 C., the amount dissolved would be considerably less, but this would still represent many times the limits indicated by Chudiakow's work.

According to McLeod and Gordon,³ agar, at 45-50 C., absorbs 0.75 volume per cent., and broth, at room temperature, about twice as much; while water, at 37 C. and 760 mm., dissolves 2.4 volume per cent. of oxygen. These liquids were oxygenated by running through them a brisk oxygen current from a cylinder for 30 minutes, and the oxygen dissolved was determined by Winkler's method. The values, it should be pointed out, are high, since they refer to the solution of the gas from pure oxygen and not from air.

It may be properly assumed that the ordinary culture mediums, especially when kept at 37 C., dissolve appreciably less oxygen than does water at 0 degree or at 20 C. At all events, the amount of dissolved oxygen which is inhibitive to anaerobic growth is considerably below the possible maximum of about 0.8 volume per cent. It probably is less than 0.05, and for some anaerobes it may even be less than 0.01 volume per cent.

The removal of this dissolved oxygen from the medium is a necessary *sine qua non* for the cultivation of anaerobes. This can be done in ways that are obvious, such as absorption, displacement, exhaustion or exclusion; and, again by methods that are more subtle. The latter are of the type in which growth occurs apparently in the presence of air.

This seeming paradox is but an illusion. Though several apparently different procedures are used, they all act by reducing, to little or nothing, the tension of the dissolved oxygen—or at least to the point where the growth of the anaerobes is possible. No matter what the appearances may be, the anaerobes remain true to type and grow only in the absence of dissolved oxygen.

The methods for securing growths, apparently in the presence of air, may be grouped under the following heads:

- (1) Microbic association or "mixed cultures."
- (2) Reducing action of chemicals.
- (3) Animal tissues.
- (4) Plant tissues.

1. *Microbic Association*.—The existence of anaerobic organisms in nature is rendered possible, as Pasteur⁴ showed, by the presence of aerobic forms which remove the free O₂ and replace it with CO₂. This principle of microbial association was enunciated by him as early as 1863, and for more than two decades the "mixed culture" of anaerobic organisms was the rule rather than the exception.

With the advent of pure cultures, this natural method was frequently utilized in one form or another. Thus, Roux⁵ (1887), having demonstrated

³ Jour. Path. & Bacteriol., 1923, 26, pp. 332-343.

⁴ Compt. rend. Acad. d. sc., 1863, 56, p. 418.

⁵ Ann. de l'Inst. Pasteur, 1887, 1, p. 57.

that *B. subtilis* by decoloring indigo blue in a sealed tube completely removed the oxygen from the medium as well as from the air, proposed to grow anaerobes in gelatine or agar, over which was superposed a layer of agar, the surface of which was inoculated with the Hay bacillus. In a way, this represents an early form of the "tandem" or partial tension method of growing certain organisms.

After confirming the work of Roger (1889),⁶ who found that a mixture of *B. prodigiosus* and *V. septique* caused fatal infection in rabbits, Penzo (1891),⁷ showed that this anaerobe would grow in the test-tube, in the presence of air, in ordinary broth, when the latter was seeded with either *B. prodigiosus* or *Proteus vulgaris*. This was but a return to the conditions known to exist in nature. Similar results were obtained by Novy (1893)⁸ when he obtained "mixed cultures" in broth, of his anaerobe with each of 4 different aerobes: Kedrowski⁹ followed (1895) with like results for a *Clostridium* and for *B. tetani*, making use of 8 kinds of aerobic organisms. Van Ermengem (1897)¹⁰ noted the same phenomenon for *B. botulinus* and *M. tetragenus*. In the following year, Scholtz¹¹ tested 4 anaerobes and more than 20 aerobes, with positive results in every case. A little later, Matzschita¹² obtained similar results with 21 aerobes and 5 anaerobes. It is unnecessary to mention subsequent observations unless it be to recall that Debrand (1903)¹³ proposed to utilize the mixed culture of *B. tetani* and *B. subtilis* as a means of preparing the toxin of the former.

In all of the foregoing instances, broth cultures were used, but the principle of "mixed cultures" holds equally true for moist surface growths. Thus, Kedrowski found that when a stab culture was made from a mixed broth culture into deep agar, the growth which developed on the surface contained the anaerobe as well as aerobe. Starting from this observation, and to make the result more striking, he inoculated the moist surface of slanted agar with the two types of organisms, and thus secured good multiplication of the anaerobes, provided the surface remained moist.

This growth on the surface of the medium, in such close proximity to the air, misled Kedrowski into believing that Pasteur was wrong in his explanation of the phenomenon of "mixed cultures." He overlooked the fact, first pointed out by Novy, that anaerobes could be grown in the water of condensation overlying a stab-culture, clearly because of displacement of air. Not realizing the extent of the gas changes going on, it seemed to him unlikely that anaerobic conditions could exist on the moist surface of the agar freely exposed to the air. Hence, he assumed that the aerobe secreted a soluble ferment-like substance which directly promoted the growth of the anaerobe.

To prove the presence of such a substance, he assumedly killed the growth of the aerobe, on agar slants, by exposure to chloroform vapor, then poured broth into the tube and inoculated it with the anaerobe. Under these conditions, the anaerobes grew well in pure culture, and apparently with free access of air. He believed that the ferment substance was dissolved out of the

⁶ Compt. rend. Soc. de biol., 1889, 41, pp. 35-38.

⁷ Atti della R. Accademia dei Lincei; Rendiconti, Ser. 4, 1891, 7, p. 210.

⁸ Ztschr. f. Hyg. u. Infektionskr., 1894, 17, p. 226.

⁹ Ibid., 1895, 20, pp. 358-375.

¹⁰ Ibid., 1897, 26, p. 47.

¹¹ Ibid., 1898, 27, pp. 132-142.

¹² Footnote 1, p. 320.

¹³ Ann. de l'Inst. Pasteur, 1900, 14, pp. 757-768.

dead cell and passed into the broth, and thus promoted growth. Although Kedrowski considered his ferment substance to be soluble, he himself was unable to grow the anaerobe in filtrates from the aerobic cultures.

The work of Kedrowski was carefully repeated by Scholtz (1898), who obtained essentially the same results as regards growth in broth, on agar slants and with filtrates. Kedrowski's theory, however, was disproved by Scholtz (1898) and by Matzuschita (1902), both of whom rightly believed that Pasteur's explanation was sufficient to account for the facts.

Von Oettingen (1903)¹⁴ attempted to obtain a "getrennte" symbiosis of *B. tetani* and *S. aureus* by inoculating each organism into broth in separate but connected tubes, the whole having an X form. Relying on the observations of Hesse¹⁵ that aerobes grown on agar consumed all the O₂ in a tube, he expected to obtain a growth of the anaerobe, but failed. He therefore concluded that the theory of Pasteur as well as that of Kedrowski did not hold, and proposed a new one, namely, that "the aerobes themselves are the nonfilterable organized ferment which makes possible the existence of anaerobes in the presence of air." The conclusion, however, was not justified, since the tubes closed with wired rubber caps were assumed, but not proved, to be free of oxygen. Moreover, the use of a broth medium does not insure complete removal of oxygen.

It will be shown later that the "getrennte" symbiosis can be readily obtained, that is to say, anaerobic organisms can be easily grown on the surface of agar plates and slants, and also in broth, when all of the oxygen is removed by separated or distant cultures of aerobes and, for that matter, by distant potatoes. This fact effectually disposes of the theories of Kedrowski and of von Oettingen. It follows that microbic association whether in intimately mixed state, or separated and at a distance, favors the growth of anaerobes, because the oxygen dissolved in the medium or present in the container is taken up by the aerobe to meet its respiratory needs.

Even an apparently dead mass of germs, killed with chloroform, can consume the oxygen dissolved in a broth and thus enable anaerobic growth. That dead cells respire was established for acetone yeast first by Telesnin¹⁶ then by Warschawsky¹⁷ and by Meyerhof.¹⁸ This, without doubt, explains the favoring action of aerobes when killed with chloroform. Actual proof of this, however, has not been supplied.

2. *Reducing Action of Chemicals.*—In the "mixed cultures," the aerobes, whether living or dead, remove the dissolved oxygen, and to that extent are reducing agents. The removal of the oxygen can, however, be effected by other means. An important first step in the cultivation of anaerobes is to heat the nutrient medium to boiling just before it is used. While primarily the purpose of this is to expel the dissolved air, it may be assumed that, at 100 C. the organic constituents of the medium reduce the residual oxygen. The result is an oxygen-free medium in which anaerobes will grow provided other factors, such as the depth of the medium, viscosity, mechanical protection, and the size of the inoculum are observed.

¹⁴ Zeitschr. f. Hyg. u. Infektionskr., 1903, 43, p. 470.

¹⁵ Ibid., 1893, 15, pp. 17-37; 183-191; 1897, 25, pp. 477-481. Arch. f. Hyg., 1897, 28, pp. 307-311.

¹⁶ Centralbl. f. Bakteriell., II, 1904, 12, pp. 205-216.

¹⁷ Ibid., 12, pp. 400-407.

¹⁸ Pflüger's Arch. f. d. ges. Physiol., 1913, 149, pp. 250-274; 1918, 170, pp. 367-427.

The success of the deep layer method with solid mediums, as first employed by W. and R. Hesse¹⁹ and subsequently developed by Liborius,²⁰ is commonly interpreted as being due to the exclusion of air. Undoubtedly, the redissolving of oxygen in the solid medium proceeds more slowly than is the case with broth. But this solution process ceases as soon as the anaerobe begins to multiply. Though imperceptible to the eye, a stream of gases (CO_2 and H_2) rises through the medium and drives out whatever air has dissolved. These gases may even displace the air overlying the medium in the tube.

A striking illustration of this was first pointed out by Novy (1893).²¹ When glucose agar tubes are freshly solidified, one or two drops of water of condensation gather on the surface. On making a stab culture into such a medium, growth takes place not only along the line of inoculation, but also in the supernatant water of condensation which becomes cloudy and shows minute gas bubbles. The cloudiness is due to large numbers of actively multiplying anaerobes, of which 3 species were tested in this manner. The same result is frequently encountered when "shake" cultures are made. Apparently the anaerobes are growing in the presence of air, but actually such is not the case. The oxygen is effectively displaced by the rising gases. Incidentally, it may be stated that such cultures in the water of condensation are rich in giant whips and long, easily demonstrable flagella.

The same phenomenon was subsequently noted by Scholtz (1898), who, however, substituted broth for the water of condensation. After making a deep stab culture in agar, the surface was covered with some broth. In this the anaerobes multiplied seemingly in contact with air. It may be added that Tarozzi²² likewise obtained growth of *B. chauvœi* and *V. septique* in the water of condensation overlying glucose agar which had been autoclaved at $2\frac{1}{2}$ atmospheres. It is hardly necessary to point out that Kedrowski's observation of growth on the surface of an agar stab culture after inoculation with a "mixed culture" is of the same order. Growth in the supernatant shallow layer of broth or water of condensation is rendered possible by the steady stream of gas which opposes solution of oxygen, and even displaces the air above the medium.

The re-solution of air in the freshly heated agar is slow, largely because of the solidity of the medium. Hence it is possible to obtain stab cultures in solid agar or gelatin mediums. But like depths of ordinary broth similarly treated as a rule will not yield cultures of anaerobes because of the relatively rapid solution of oxygen in the clear and mobile liquid.

If however, the viscosity of the glucose broth is changed by the addition of 2-10% of gelatine, growth can easily be obtained at 30-37 C. in the liquid medium, as was first shown by Novy²³ (1893). This observation was confirmed by Braatz (1895),²⁴ and by Matzuschita (1902), while Scholtz (1898) found that the addition of some melted agar or even of a small lump of agar likewise promoted the growth of anaerobes. The viscosity of the liquid retards the re-solution of the oxygen, and it is probable that the large colloidal surface present, as well as the reducing sugar, promotes the reduction of any dissolved oxygen. It may be pointed out that the addition of a little

¹⁹ Deutsche med. Wchnschr., 1885, 11, p. 214.

²⁰ Ztschr. f. Hyg. u. Infektionskr., 1886, 1, pp. 115-177.

²¹ Centralbl. f. Bakteriöl., 1893, 14, p. 590; Ztschr. f. Hyg. u. Infektionskr., 1894, 17, p. 219.

²² Atti della R. Accademia dei Fisiocritici in Siena, Ser. 4, 1906, 18, p. 408.

²³ Footnote 21, pp. 590-598; 219-220.

²⁴ Centralbl. f. Bakteriöl., I, 1895, 17, p. 739.

agar to dilute serum, as is well known, favors the growth of spirochetes. The organisms in this case are to be found in the cloudy agar suspension and not in the clear supernatant liquid. The oxygen content of the former is undoubtedly lower than that of the clear liquid.

Kitt²⁵ obtained cultures in broth provided it was used in quantities of 0.5-1 liter and was heavily inoculated, preferably with 1-3 c.c. of the inoculum. Although he did not view this procedure as comparable to the deep layer method, it must nevertheless be looked on as such.

A heavy inoculum materially assists in bringing about an oxygen-free zone in which the organism can commence to grow. Thus, Scholtz showed that a small mass of culture, pinhead in size, when introduced into a tube of broth first enlarged and then gave a growth throughout the liquid.

The mechanical protection caused by precipitates (coagulated broth) and by added insoluble matter such as agar (Scholtz) or cotton (Hata), powdered metals, elder pith, etc., favors the removal of oxygen in the occluded liquid, and thus promotes the initiation of anaerobic growth. The same explanation may hold, in part at least, for the classical experiments in infection of animals by placing subcutaneously bits of paper, sand or agar impregnated with heated spores of *B. tetani* or *V. septique* (Vaillard and Rouget,²⁶ Besson,²⁷ Roncali²⁸).

The reducing action of organic substances was recognized very early, but Pasteur (1863)²⁹ was the first to show that sterile infusions, saw-dust, urine, milk and blood took up oxygen and gave off CO₂. About thirty years later, Hesse³⁰ (1893) demonstrated that ordinary agar, if alkaline, showed the same gas exchange. Somewhat later, Pfuhl (1907)³¹ obtained a similar result with a liver mash. All mediums may be said to respire, that is, they take up O₂ and give off CO₂. Some, as for example blood agar, produce a marked gas exchange while plain agar is much less active in this respect.

Recognizing the importance of removing dissolved oxygen, many efforts were made to accomplish this by the addition of reducing substances. The first substance of this kind to be used was sugar (Pasteur). Liborius (1886)³² found that the addition of 1-2% of glucose greatly improved the medium. Sugar is not only a valuable source of energy for the anaerobes, but by reducing the dissolved oxygen, it directly promotes the initial multiplication. Consequently, it has ever since been looked on as an almost necessary constituent of anaerobic mediums.

A variety of reducing substances have been tested. Indigotin was used by Liborius (1886). Kitasato and Weyl³³ (1890) examined a large number of compounds, and among these sodium formate and sodium indigo sulphate gave the best results. Other workers obtained more or less favorable results with sulphides (Trenkmann, Hammerl), sulphites (Beijerinck, Hata), FeSO₄ (Arloing, Hata), metallic iron (Wrzosek, Hata), platinum sponge (Pfuhl) and coal, coke and charcoal (Wrzosek). Incidentally, it may be added that charcoal gives off CO₂ when immersed in water through which a current of air is passed (Stoklasa³⁴).

²⁵ Ibid., I, 1895, 17, pp. 168-171.

²⁶ Ann. de l'Inst. Pasteur, 1893, 7, p. 767.

²⁷ Ibid., 1895, 9, p. 192.

²⁸ Riforma med., July, 1893, 9, p. 175.

²⁹ Footnote 4, pp. 734-740.

³⁰ Ztschr. f. Hyg. u. Infektionskr., 1893, 15, pp. 183-191.

³¹ Centralbl. f. Bakteriolog., I. O., 1907, 44, pp. 378-383.

³² Footnote 20, p. 168.

³³ Ztschr. f. Hyg. u. Infektionskr., 1890, 8, pp. 41-47.

³⁴ Ztschr. f. physiol. Chem., 1907, 50, p. 340.

A useful substance in this regard is litmus. As early as 1843, Helmholtz³⁵ noted that it was reduced in the presence of decomposing matter, and for that reason he used it as a reagent for putrefactive changes. It was used as an indicator of the reaction in cultures of molds by Leber³⁶ (1882), and of bacteria by H. Buchner³⁷ (1885). However, the first to employ it to show the reducing action of pure cultures of aerobic and anaerobic bacteria was Cahen³⁸ (1887).

As pointed out, Novy (1893) showed that anaerobes could be grown in a 10-15% gelatine medium, 4-5 cm. in depth, containing 2% glucose, with or without litmus, though the best results were obtained when litmus was present. When such tubes are inoculated and placed at 37 C., the litmus becomes reduced, gas is evolved, and rapid multiplication takes place, notwithstanding the fact that the medium is liquid and apparently in contact with air. Excellent cultures were obtained with freshly heated broth, 7-8 cm. in depth, containing 2% each of gelatine, glucose and peptone, with or without litmus. Ever since then the litmus glucose gelatine medium has been used as a convenient means of growing anaerobes for stock purposes. Such cultures, though developed in the same manner as aerobes, remain viable for years.

It may be mentioned that glucose litmus gelatine when freshly prepared is colored evenly and throughout. After standing for some days at room temperature, the lower half of the medium in the tubes becomes decolorized, showing that the oxygen is being reduced as fast as it diffuses downward below a certain level.

Attempts have been made to obtain growths of anaerobes on Petri plates, in the presence of air, using for this purpose mediums which contained reducing substances. These trials, however, have invariably failed, clearly because oxygen can be absorbed from the air by the shallow layer of agar about as fast as it can be reduced. Tarozzi,³⁹ by using an alkaline glucose agar autoclaved at 2 to 2.5 atmospheres for 5 to 10 minutes, obtained plate cultures of 12 anaerobes. It was necessary for him, however, to have the medium about 1 cm. deep, and to use a modified Petri dish with ground contact surfaces and a paraffin seal to exclude oxygen. In this case the oxygen, above as well as in the medium, was presumably taken up by the reducing substances present.

As will be shown later, failure has likewise been the result in similar attempts at plate cultivation with mediums enriched with extracts of tissues, or with finely divided suspensions of organs.

3. *Animal Tissues*.—It was an obvious procedure to use small pieces of infected tissue in starting a culture of the organism present, whether aerobic or anaerobic. The sole object in view was to isolate the organism. That the tissue itself could exert a direct favoring action was not recognized for some time. Illustrative of the early use of both animal and plant tissue in bringing about anaerobic growth may be mentioned the work of Gaffky⁴⁰ (1881). He obtained cultures of the bacillus of malignant edema by placing a piece of liver containing the organism inside of a boiled potato, the opening

³⁵ Arch. f. Anat. Physiol. u. Wissensch. Med., 1843, p. 457.

³⁶ Berl. klin. Wchnschr., 1882, 19, p. 163.

³⁷ Arch. f. Hyg., 1885, 3, pp. 417-419.

³⁸ Ztschr. f. Hyg., 1887, 2, pp. 386-396.

³⁹ Footnote 22, pp. 401-415.

⁴⁰ Mittheil. a. d. kaiserl. Gsndtsamte. Berlin, 1881, 1, p. 91.

being closed up with the potato mash. Even a second generation was thus obtained.

W. and R. Hesse, in 1885, obtained cultures of this organism, apparently in the presence of air, by placing bits of infected tissue in 1% agar or 5% gelatine, the growth taking place at room temperature. As is well known, this use of solid mediums was developed by Liborius into the so-called deep layer method for cultivating anaerobes.

Tizzoni, Cattani and Baquis⁴¹ (1890) drew attention to the fact that the tetanus bacillus could be grown, apparently as an aerobe, in clotted rabbit blood. The change in the color of the blood which they noted was indicative of reduction. This result with normal blood may be considered as the first example of normal tissue used to promote anaerobic growth. It may be added that von Hibler⁴² (1899) similarly grew a number of anaerobes in fresh rabbit blood, and also in blood which had been coagulated by steaming. Tizzoni also obtained so-called aerobic cultures by placing material from infected spleen in ordinary mediums, thus confirming the work of Hesse.

In 1890, Smith⁴³ introduced the fermentation tube which previously had been used in urinalysis. He then pointed out that small pieces of infected liver or spleen, when placed in the closed end, gave growths of apparently anaerobic organisms. This was essentially in line with the work of Hesse and of Tizzoni, who had shown that infected tissues planted in a deep medium gave rise to cultures. No tests were made at that time with the common obligative anaerobes or with normal tissue.

Of more importance, however, is the fact, brought out by Smith⁴⁴ for the first time in 1899, that small pieces of sterile spleen, liver or kidney, taken from normal animals, when placed in the closed end of the fermentation tube promoted the growth of *B. tetani*. With the exception of the work of Tizzoni with normal blood, likewise on *B. tetani*, this is the first instance in which normal tissues were used for this purpose.

A similar observation was made by Kitt⁴⁵ (1902), who found that pieces of sterile muscle promoted the growth of *B. chauvoei*. Independently, Tarozzi⁴⁶ (1905) worked out the tissue method and showed its applicability in the cultivation of 14 anaerobes. He used bits of liver, spleen, kidney and other tissues from normal animals. Growths of anaerobes, apparently in the presence of air, were obtained by placing such tissue in ordinary broth tubes, or in the water of condensation on slanted agar. Similarly, serum and ascitic fluid which by themselves gave no growth of the anaerobes promptly did so on the addition of bits of tissue. Incidentally, it may be stated that Ori⁴⁷ (1905) had previously shown that anaerobes which would not grow in heated or unheated serum did so promptly on the addition of a piece of liver. Noteworthy also is the fact, brought out by Tarozzi, that broth containing coagulated albumin, made by heating the meat extract in the autoclave at less than one atmosphere of pressure, gave a growth of the anaerobes, whereas if filtered, it gave none. The favoring action of precipitates has already been referred to.

⁴¹ Beitr. z. path. Anat. u. z. allgem. Path., 1890, 7, p. 597.

⁴² Centralbl. f. Bakteriologie, I, 1899, 25, p. 603.

⁴³ Ibid., 1890, 7, pp. 502-506.

⁴⁴ Jour. Boston Soc. Med. Sc., 1899, 3, p. 343.

⁴⁵ Monatsh. f. prakt. Tierheilk., 1902, 13, p. 174.

⁴⁶ Atti d. R. Accad. Fisiocritici in Siena, Ser. 4, 1904, 16, pp. 319-329; 1905, 17, pp. 105-124; pp. 225-258; Riforma med., 1905, 21, pp. 146-150; 182-184; 208-211; Centralbl. f. Bakteriologie, I. O., 1905, 38, pp. 619-624.

⁴⁷ Atti d. R. Accad. d. Fisiocritici in Siena, Ser. 4, 1905, 17, p. 54.

The work of Tarozzi was confirmed within a month by Ori,⁴⁸ and somewhat later by Grixoni,⁴⁹ and then by Wrzosek.⁵⁰ The latter emphasized the importance of the relative amounts of broth and tissue used. He showed further that the proper mixture could be sterilized at 120 C. for 15 minutes without impairing the property of promoting anaerobic growth in the presence of air. Harrass⁵¹ (1906) arrived at the same result by heating the broth and brain or liver mixture at 100 C. for 1½-2 hours. Although Tarozzi previously had obtained a like result with autoclaved, coagulum-containing broth, he did not obtain good results with heated tissue broth, probably because of insufficient tissue. It should be pointed out that heated tissue had been used even earlier than this by von Hibler⁵² (1899), who not only used blood coagulated by heat, but also resorted to steamed brain mash for the purpose of growing anaerobes in the presence of air. He did not, however, tube the latter medium, but used it in flasks, thus actually making use of the deep layer method, just as Kitt did when he used large volumes of broth for his so-called air cultures. The recent use of meat mash for cultivation of anaerobes, in the presence of air, is in line with this early work of von Hibler. It had been used years before, however, for cultivation in partial vacuum by Besson.⁵³

A passing mention may be made of the use of milk for the cultivation of anaerobes, apparently in air. Klein (1895)⁵⁴ employed this medium for the isolation of his anaerobe, but placed it over pyrogallate. Apparently the first to use it in the presence of air was von Hibler,⁵⁵ who, however, obtained only poor growths. Tarozzi⁵⁶ found that the growth was not as rich as in tubes with broth and tissue, but he did get positive results with 10 out of 12 strains of anaerobes. The milk was sterilized at 120-125 C. Smith, Brown and Walker⁵⁷ had no difficulty in obtaining active multiplication of 12 strains in sterile milk contained in fermentation tubes. The reducing action of milk is marked and may be due to lactose, although Pasteur⁵⁸ (1863) pointed out that the fat probably took up the oxygen.

Though the broth-tissue mixtures, heated or unheated, gave growths of anaerobes in tubes or flasks, apparently in the presence of air, all attempts to make use of like mediums with agar for plating purposes failed. Thus, Ori⁵⁹ tried to obtain plate cultures by adding to the agar a glycerol extract of guinea-pig liver; Harrass attempted it by mixing the brain or liver mash with the agar, which was then either slanted or poured into plates. On the slanted mixture, no growth was obtained except in the water of condensation or in broth if it was poured on the surface. The agar plates likewise gave no growth unless covered by 2-3 mm. layer of broth. He also tried plate cultivations with mixtures of animal and plant tissues to which reference will

⁴⁸ Ibid., pp. 53-55; 169-174.

⁴⁹ Gior. med. del. R. Esercito, 1905; Centralbl. f. Bakteriöl., I. Ref., 1906, 38, p. 17.

⁵⁰ Wien. klin. Wehnschr., 1905, 18, pp. 1268-1270; München. med. Wehnschr., 1906, 53, p. 2534; Centralbl. f. Bakteriöl., I. O., 1907, 43, pp. 17-30; 44, pp. 607-617; 1910, 53, pp. 476-477.

⁵¹ München. med. Wehnschr., 1906, 53, pp. 2237-2240.

⁵² Footnote 42, p. 605.

⁵³ Ann. de l'Inst. Pasteur, 1895, 9, p. 181.

⁵⁴ Centralbl. f. Bakteriöl., I, 1895, 18, p. 743; 1897, 22, p. 114; 1898, 23, p. 542.

⁵⁵ Footnote 42, p. 604.

⁵⁶ Atti d. R. Accad. d. Fisiocritici in Siena, Ser. 4, 1905, 17, p. 118.

⁵⁷ Jour. Med. Res., 1905, 14, p. 202.

⁵⁸ Footnote 4, p. 736.

⁵⁹ Footnote 47, p. 169.

be made later. Only recently, McLeod and Gordon,⁶⁰ apparently unaware of Ori's work, reported a similar failure in their attempts to grow anaerobes in the air on plates heavily charged with a catalase obtained from rabbit liver.

The way in which tissues, heated or unheated, favor the growth of anaerobes has been the subject of some speculation. The fact that the tissue may supply useful nutritive substances to the medium is evident, but that in itself is not sufficient to explain the phenomenon. The theory that first suggests itself is that the tissues absorb and remove the oxygen from the medium. Tarozzi, while recognizing this possibility, endeavored to show that the simple removal of free oxygen from the medium was not sufficient cause to bring about development of anaerobes. He believed that oxygen inhibited anaerobes, not by direct toxic action, but indirectly by oxidizing the nutritive substances which thus became unassimilable. He imagined that such oxidized products, in the presence of reducing substances, became reduced and were then utilizable for growth. This view was shared by Ori.

According to Wrzosek, the favoring substance or property was present in animal and plant tissues, in seeds, charcoal, coal, coke, chalk, zinc and iron. Consequently, the apparent growth in air was due to a reducing substance. Guillemot and Szczawinska⁶¹ (1908) pointed out that the removal of oxygen by the reducing substance was the true explanation. McLeod and Gordon concluded that "anaerobes cannot tolerate more than very slight concentrations of oxygen because they produce H_2O_2 as soon as oxygen is available and being very sensitive to this agent they die." Hence, referring to Tarozzi's work, they suggested that the tissues used may have catalase, which, by reducing the hypothetical H_2O_2 , thereby promotes the growth of anaerobes.

The favoring action of tissues, therefore, has been ascribed to the presence of either special nutritive substances, or of reducing agents. For the latter, three possibilities have been put forth: (1) reduction of the inhibiting H_2O_2 , (2) reduction of the oxidized nutritive substances, and (3) reduction of the dissolved and directly toxic oxygen. The last explanation is undoubtedly the true one, as will be shown by the work on plant tissues.

4. *Plant Tissues*.—As already mentioned, Gaffky made use of the boiled potato to cultivate the bacillus of malignant edema. Apparently, with the exception of von Hibler's⁶² rice-salt medium, no further use of vegetable material was made until Ori,⁶³ at the suggestion of Sclavo, tested plant tissues to ascertain whether they were capable of favoring the growth of the 3 common anaerobes in the presence of air, the same as had been done with animal tissues by Tarozzi. His best results were obtained with broth containing sterile raw potatoes, carrots and turnips. Apples and lemons gave no growth, while orange and corn sprouts gave cultures only of the bacillus of malignant edema. The tubes when heated in the water-bath for 10 minutes likewise gave rich growths, except in the case of *B. tetani*.

Tarozzi⁶⁴ confirmed the work of Ori, but he considered the growth to be inferior to that obtained with animal tissues. He conceded, however, the identity of action of plant and animal tissues. Wrzosek attempted to repeat the work with raw potato, but because of contamination, he resorted to

⁶⁰ Footnote 3, p. 337.

⁶¹ Compt. rend. Soc. de biol., 1908, 64, pp. 171-173.

⁶² Footnote 42, pp. 604 and 609.

⁶³ Footnote 47, p. 172.

⁶⁴ Atti d. R. Accad. d. Fisiocritici in Siena, Ser. 4, 1905, 17, p. 239.

sterilization of the broth-potato tubes in the autoclave at 120 C. for 15 minutes. He obtained good cultures when the relative amount of potato and broth was 1:5 or 1:10. He explained the favoring action as being due to the presence of a reducing substance, but left it to be inferred as to what was reduced. Harrass attempted to make a semisolid medium for plating anaerobes in air by combining brain or liver mash with starch paste or potato mash. The latter gave him a diffuse growth but no colonies.

The use of the potato for the culture of anaerobic organisms has recently been revived by Avery and Morgan.⁶⁵ Adopting the theory of McLeod and Gordon as to the potential formation by anaerobes of H_2O_2 and its destruction by catalase, they conclude "that anaerobic organisms fail to grow in the presence of air not because atmospheric oxygen as such is a direct poison to the cell, but because of toxic peroxides which are produced whenever the oxygen of the air combines with auto-oxidizable substances of the bacterial cell." To quote further: "If this assumption is correct, then the aerobic growth of obligate anaerobes in the presence of plant tissue finds partial explanation at least in the fact that the peroxide formed by auto-oxidation is rapidly broken up by the catalase and peroxidases of the plant tissue."

It will be seen, as regards the favoring action of plant tissues, that, apart from the question of the presence of growth-promoting factors, 3 distinct views have been presented. These are identical with those given for animal tissues. They essentially have, in common, a reducing substance which, (1) regarded as a catalase, effects reduction of the hypothetical hydrogen peroxide; or (2) acts on the supposedly oxidized substances of the medium and makes the latter suitable for growth; or (3) brings about the reduction of the free dissolved oxygen. The work which is to be presented will furnish conclusive evidence that the plant tissue favors the growth of anaerobes by complete removal of the oxygen that may be present, and that its action is in nowise different from that of the aerobic organisms.

POTATO RESPIRATION

Since the potato, more than any other plant tissue, has been used to favor the growth of anaerobes, it was selected for the study of the reaction involved. The marked respiratory changes shown by aerobic organisms made it reasonable to believe that the potato likewise consumed oxygen and gave off CO_2 . It was necessary, however, to prove that such was the case. This was done by applying the methods which have been developed in this laboratory in the investigation of microbial respiration. They proved to be effective in solving the problem of the gas changes produced by the potato. By exact quantitative analyses it was possible to demonstrate that marked aerobic and anaerobic respiration took place.

⁶⁵ Jour. Exper. Med., 1924, 39, pp. 289-302.

Methods.—In this work, the potato was used in the form of cylinders, 8-10 mm. in diameter and 40-50 mm. in length, which were made by means of brass or nickel-plated cork-borers. Each borer was provided with a somewhat longer glass rod, one end of which was flattened. The rod served as a piston in ejecting the potato cylinder. The thoroughly cleaned borers, each with its piston, were placed in an iron box and sterilized in the dry-heat oven at 200 C.

The preparation of raw, sterile potato cylinders is a simple matter. After previous cleansing with a brush, the potato was placed in HgCl_2 solution (1:1,000) for at least half an hour. A number of ordinary potato knives were sterilized in the flame and placed on a convenient support. The potato was then held vertically, and the upper portion was pared by a single cut, the sterile knife being held so as to make a truncated cone. With a second knife, a horizontal cut was then made, through the pared surface, thus completely removing the end portion with the least possible chance of infection. As a further precaution, with a third knife, another thin horizontal layer was removed. The potato was then inverted, and the same operation was applied to the other end. The median portion of the potato was not pared.

A number of Petri dishes, each containing several thicknesses of filter paper, were previously sterilized. The potato was then placed so that one of the cut ends rested on the sterile paper in the bottom of a Petri dish. The sterile filter paper served to take up such fluid as was squeezed out of the potato, and thus restricted the possibility of contaminating the cut surface.

With a sterile cork-borer, a cylinder of potato was punched out and then ejected into a sterile test-tube. A second and then a third cylinder was punched, each with a separate borer, and transferred to the same sterile test-tube. The usual procedure was to place 3 cylinders in each tube, but with very large potatoes 1 or 2 often sufficed to give the desired weight.

With each of the borers which had been used, a second cylinder was punched, and this set, which corresponded exactly to that in the test-tube, was then weighed, thus giving the weight of the potato in the sterile tube. Of course, this weight can be determined by weighing the tube before and after the introduction of the cylinders.

Frequently, as a control of the sterility, a corresponding cylinder was placed in another sterile test-tube. This was then covered with sterile broth, a supply of which was always kept in a globe funnel. Similarly, at the close of a respiration experiment, sterile broth sufficient to cover the cylinders was always introduced into the tube, which was then set aside in the incubator for 48 hours. The presence of any contaminating aerobic or anaerobic organisms was thus revealed. The experiment was discarded whenever any contamination was found. As a matter of fact the raw potato cylinders were almost invariably sterile.

The raw sterile potato was usually exposed directly to the air in the tube. In some experiments, however, the cylinders were covered with sterile distilled water, plain broth, glycerol broth or glucose broth. A number of tests were made with potato cylinders sterilized in the autoclave at 120 C. for 15 minutes.

The plugged sterile tubes which received the potato cylinders were usually of the *h* type (fig. 1B, Part I). These were used because of their large air capacity. They were especially useful when it was desired to show the effect of potato or of aerobes on the growth of anaerobes.

The tubes were attached to the manometers in the usual way. The cotton plug was trimmed, then flamed and pushed within the tube, after which the

tube was attached to the rubber stopper, which had been treated with glycerol, on the end of the manometer. The latter, with the attached tube, was then placed in the hot-room, and, after 2 hours, cocks 1 and 3 were opened, and the manometer was equilibrated. The cocks were then closed and secured in place by rubber bands (fig. 4, Part I).

The use of the rubber stopper in connecting the *h*-tube with the manometer, though convenient, is certain to introduce an error in the manometric readings and in the analyses. This is because of the solubility of CO₂ in rubber. The error increases with the tension of the CO₂ present and with the duration of the experiment. The effect of such loss of CO₂ is to increase a negative pressure, and to lessen a positive pressure.

When it was desired to expose the potato cylinders to an atmosphere of pure oxygen or nitrogen, the tubes were evacuated and filled with these gases in the manner described in Part I.

In special cases, the tubes were placed in Novy jars which were used with or without manometers, according to the purpose in view.

The withdrawal of the gas sample from the tube or jar was effected in the usual way. The gas was drawn either into the buret or into the sampler (fig. 14, Part I). The latter is to be preferred whenever duplicate analyses are desired. It is particularly useful in drawing gas from the anaerobe jar.

Raw Potato in Air.—The respiration in air of the sterile raw potato cylinders was studied in *h*-tubes which were attached to manometers. In 10 experiments of this kind, 46 tubes were used, each experiment being made with from 3 to 9 tubes. These tests varied as regards the effect of (1) the age of the potatoes, (2) the duration of treatment with HgCl₂, (3) the amount of potato used, and (4) different temperatures.

Table 1, which primarily is intended to bring out the effect of different temperatures on the rate of respiration, may be viewed as typical of the changes involved in the other experiments.

The first effect to be noted is, as in the case of aerobic organisms, the production of a negative pressure. The exact maximal negative pressure which developed could be ascertained only by making observations at close intervals of time. In this experiment, the observed negative maxima ranged from —10 to —22, the observations being made at 19 or 41 hours.

This phase indicated active aerobic respiration and that the oxygen consumption was slightly greater than the CO₂ production. The respiratory quotient at this stage should be somewhat less than 1. Another explanation of the negative pressure could be offered on the assumption that basic products were formed which removed some of the CO₂ which was formed. This, however, did not seem to be the case. The chief cause of the negative pressures in this experiment was the absorption of CO₂ by the rubber stopper (compare table 4).

The negative pressure did not reach a constant level, but, as in the case of facultative anaerobic bacteria, it sooner or later began to fall, and eventually a positive pressure developed. It will be seen from table 1 that in 87 hours one of the tubes showed a pressure of +101 mm. (uncorrected). There was every reason to believe that, had the experiment been carried through a longer period, a much higher pressure would have been obtained, the result depending on the amount of potato used, the temperature and the air volume of the tube.

TABLE 1
RESPIRATION OF RAW POTATO AT DIFFERENT TEMPERATURES

Temp., C.	31			34			39		
Tube No.	1	2	3	4	5	6	7	8	9
Potato, Gm.	15	14.9	11.4	11.9	14.7	10.4	13.5	12.5	12.4
Hrs.									
Equil., 0.....	0	0	0	0	0	0	0	0	0
2.....	-1	-1	-3	-3	-3	-1	-3	-1	0
4.....	3	1	4	5	3	2	7	2	-4
19.....	-10	-11	-10	-13	11	10	14	8	9
41.....	+30	+15	+4	+7	-9	12	-12	22	21
46.....	+41	+16	+1
54.....	47	30	+9	-1	-12	-13
65.....	70	54	29	+13	+11	+6
69.....	+80
75.....	+40	+23
77.....	78	29	15
87.....	+101	45	35
88.....	+46
90.....	+38
Corr. obs. man.	41.9	82.2	103.6	16.2	40.5	46.1	1.0	23.3	38.5
Calc. real man.	35.9	95.2	123.6	15.9	47.5	43.3	12.6	27.0	40.8
Analyses									
CO ₂	24.76	29.51*	32.71*	22.68	25.96	25.54	22.34	23.93	25.18
O ₂	0.0	0.80	0.0	0.03	0.0	0.0	0.04	0.0	0.19
N ₂	75.24	69.69	67.29	77.29	74.04	74.46	77.62	76.07	74.63
	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
Corr. Analyses									
CO ₂	26.01	33.469	38.421	23.194	27.713	27.111	22.748	24.864	26.668
O ₂	0.0	0.907	0.0	0.03	0.0	0.0	0.041	0.0	0.201
N ₂	79.04	79.04	79.04	79.04	79.04	79.04	79.04	79.04	79.04
	105.05	113.410	117.461	102.264	106.753	106.151	101.829	103.904	105.909
Real resp. quot.	1.241	1.670	1.834	1.104	1.323	1.294	1.088	1.187	1.285

* Sample analyzed was 2.5-2.8 c.c.

The swing in pressure from negative to positive showed that anaerobic respiration was taking place. It is likely that this type of respiration was initiated as soon as the amount of oxygen was appreciably decreased. It became particularly evident when all of the oxygen was removed. Conceivably, anaerobic respiration is continual in the central portion of the potato cylinder, where it would seem that free, intercellular oxygen must be absent.

Examination of table 1 shows that variations in the temperature markedly affected the respiratory change. At 31 C., the aerobic phase was of shorter duration than at 39 C. Similarly, the anaerobic phase was more pronounced at 31 C. than at 34 C. or at 39 C. This, perhaps, was just what might be expected of the potato, which sprouts in the soil when the temperature is appreciably below 30 C. The optimal temperature for the enzymes concerned in the respiratory process is therefore at or below 30 C.

The analyses are of special interest. Five of the tubes showed an entire absence of oxygen. In tubes 4 and 7, the amount of oxygen was clearly within the experimental error, while in tubes 2 and 9, an error in sampling may be assumed.

It will be seen that the analyses showed that the CO_2 in each tube was in excess of 22%, and that the amount increased with the duration of the experiment. Furthermore, the highest yield of CO_2 was in the tubes kept at 31 C. The recalculation of the results to the nitrogen basis (79.04) appreciably increased the analytical values. Thus, in tube 3 an actual yield of 38.4% of CO_2 was indicated. In these tests, hydrogen was not looked for, but subsequent trials showed the absence of any combustible gas.

The calculated real manometric readings in 6 of the tests showed satisfactory agreement with the corrected observed values. The divergence for tubes 2, 3 and 7 may be due in part to errors incidental to sampling.

Attention may be called at this point to the values for the real respiratory quotients derived from the corrected analytical data. The quotient, at a given temperature, increased with the duration of the experiment. This, of course, was a consequence of the progressive increase in the amount of CO_2 produced. It will be noted that the quotients were highest at 31 C. and lowest at 39 C. Special experiments dealing with the respiratory quotient will be taken up later.

It would be of some interest to know the effect of aging on the respiratory rate. Conceivably, potatoes which are 6-8 months old, and which tend to sprout in the room, are richer in enzymes. In the beginning of this study, old potatoes were used, but it soon became necessary to use the new ones because of the market supply. At first, it was believed that there was an appreciable difference in the results, but further work did not bear out this view. It may be added that in the experiments to be described only new potatoes were used.

Another point which received some attention was the question of the effect of HgCl_2 which was used in the preliminary treatment of the potato. Experiments showed that there was essentially no difference between treated and untreated potatoes, or between those that were soaked in HgCl_2 for half an hour and those that were soaked for 18 hours.

The amount of the potato used is an important factor in these experiments; likewise the surface area presented by the potato cylinders. In the experiments to be given, the influence of the weight will readily be seen.

TABLE 2
RESPIRATION OF POTATO, RAW AND AUTOCLAVED, AT 34 C.

		Raw		Autoclaved	
Tube No.		1	2	3	4
Potato, Gm.		7.1	6	5.9	5.4
Hrs.					
Equil., 0.....		0	0	0	0
1.....		-4	-3	-2	-2
3.....		4	3	2	3
18.....		4	2	2	3
29.....		7	5	4	3
41.....		7	6	4	3
48.....		6	7	3	3
55.....		-5	6	3	3
66.....		+1	6	2	3
70.....		+4	6	-3	3
90.....		...	-1	...	3
99.....		...	+2	...	3
103.....		...	4	...	5
116.....		...	11	...	5
121.....		...	+14
138.....		-7
Analyses					
CO ₂		21.11	22.85	0.45	0.69
O ₂		0.30	0.78	20.33	19.63
N ₂		78.59	76.37	79.22	79.68
		100.00	100.00	100.00	100.00

Effect of Autoclaving.—Repeated experiments having shown that the raw potato cylinders exhibited aerobic and anaerobic respiration, it was desirable to ascertain whether this change persisted after steam sterilization. For this purpose, sterile cylinders were placed in 4 *h*-tubes. Two of the tubes were reserved as controls and 2 were heated in the autoclave at 120 C. for 15 minutes. The 4 tubes were then attached to manometers, placed in the hot-room at 34 C., and equilibrated. The result of this experiment is given in table 2. It may be added that 4 similar experiments gave essentially the same result.

On comparing tables 1 and 2, it should be noted that the weight of the potatoes varied in the two experiments. In fact, the amounts taken

for this experiment were less than one-half those used in the former. Consequently, the control tubes with raw potato showed a lower negative maximum, and the negative phase was prolonged for 2 and even 3 days. Because of the smaller quantity of potato, the oxygen consumption was not as rapid, and the analyses at 70 and 121 hours showed that the utilization was still incomplete. For the same reason, the positive pressures were low compared with those recorded in table 1.

The effect of autoclaving was seen in tubes 3 and 4. Practically no change in the manometric readings took place for 3 or 4 days. The initial change within the first hour was due to insufficient time being allowed before equilibration.

The analyses are particularly instructive. The tubes with the heated potato gave only about 0.5% of CO_2 , and it is probable that the major part of this was merely given off by the potato. A potato cylinder contains an appreciable amount of CO_2 (0.25 c.c. per gram), and some of this would tend to pass out. An analysis of a special control should have been made at the time of equilibration. However, the result as it shows that very little oxygen was taken up by the heated potato. Auto-oxidation of dead matter is a slow process. On the other hand, the raw potato exhibited its characteristic respiratory change, since practically all of the oxygen was consumed and more than 21% of CO_2 was returned.

Respiratory Quotient.—It was incidentally pointed out in connection with table 1 that the respiratory quotient increased with the duration of the experiment because of the additional CO_2 produced by the anaerobic process. The true respiratory quotient is to be deduced only from the CO_2 which is actually derived by the consumption of free oxygen. It therefore is an expression of aerobic changes, and should be constant. When the aerobic condition is followed by the anaerobic phase, in which CO_2 is being continually produced, the respiratory quotient will be a variable, depending on how much additional CO_2 has been made.

It seemed desirable to ascertain the true respiratory quotient of potato and to bring out clearly the added effect of the anaerobic phase. With that object in view, the following experiment was made.

Sterile raw cylinders were placed in each of 4 *h*-tubes which were then connected with manometers, placed at 34 C. and equilibrated. The analyses were made at sufficiently long periods in order to obtain the desired results. It will be seen from table 3 that the first analysis was made when a negative pressure was present. The 4th tube was analyzed when it showed a positive pressure of +134 mm. of Hg.

As usual, the analytical results were reduced to the nitrogen basis (79.04). The corrected value for CO_2 less 0.03, the amount present in air, gave the real gain in CO_2 . This value, however, may be somewhat low because of the loss of CO_2 by solution in the rubber stopper. Similarly, the corrected value for oxygen, subtracted from 20.93 gave the real loss in O_2 . Dividing the former by the latter gave the real respiratory quotient, uncorrected, however, for any CO_2 taken up by the potato.

Tube 1, which contained 1.3% O_2 , gave a respiratory quotient of 0.957, which approximates 1, the theoretical quotient for a carbohydrate. Tube 2, with a manometer at +10, gave a quotient of 1.048, while

TABLE 3
RESPIRATORY QUOTIENT OF RAW POTATO AT 34 C.

Tube No.	1	2	3	4
Potato, Gm.	14.8	15.6	15.3	18.8
Hrs.				
Equil., 0.....	0	0	0	0
12.....	-3	-2	-4	-5
18.....	-4	-3	-3	0
36.....	...	+10	+33	+42
60.....	+80	97
84.....	+134
Corr. obs. man.	4.0	10.1	81.9	136.9
Calc. real man.	5.9	7.0	88.6	137.7
Analyses				
CO ₂	18.97	21.39	29.82	33.87
O ₂	1.32	0.35	0.0	0.0
N ₂	79.71	78.26	70.18	66.13
	100.00	100.00	100.00	100.00
Corrected analyses				
CO ₂	18.81	21.603	33.585	40.482
O ₂	1.31	0.354	0.0	0.0
N ₂	79.04	79.04	79.04	79.04
	99.16	100.997	112.625	119.522
Real resp. quot.	0.957	1.048	1.603	1.933

tube 3, with the pressure of +80, gave 1.603. Tube 4, with the highest reading of +134, showed a quotient of 1.933, which was actually twice the value given by tube 1. Since the anaerobic respiration can develop a pressure of +200, if not more, in tubes of the type used, it follows that considerably higher quotients could be obtained. Such high quotients will be found in table 8.

The large amount of CO_2 formed under these conditions cannot be due to the action of acid products on the NaHCO_3 which may be present in the potato. For, it will be shown later, that the total of free and combined CO_2 per gm. of raw potato amounts only to about 0.25 c. c. at 0 degree and 760 mm. It follows from this experiment that anaerobic

respiration is indicated, in the absence of carbonates, whenever a micro-organism or tissue gives a respiratory quotient which is appreciably higher than 1. Here the anaerobic phase was superposed on the original aerobic respiration.

In order to bring out more clearly the fact that the respiratory quotient, under aerobic conditions, approximated 1, the foregoing experiment was modified by replacing the *h*-tubes with large Novy jars which had a net air capacity of from 1,600 to 2,500 c.c. Each jar received 2 tubes (20 x 150 mm.) containing a total of about 22 gm. of sterile potato cylinders. These tubes were, of course, sterile and plugged with cotton. On the bottom of the jar, 1 c.c. of distilled water was placed to supply the requisite aqueous tension. The jars were then sealed, attached to manometers as described in Part I, (fig. 6) and placed at 34 C. The experiment was made in triplicate. The results are given in table 4.

The jars were analyzed at the end of 3, 5 and 10 days, respectively. Because of the large volume of air present, the oxygen was not completely consumed. Even at the end of 10 days, there were 5.6% present, whereas in the case of the *h*-tubes, used in the preceding experiment, the oxygen was practically removed in 36 hours. Consequently, the potato cylinders in the jars were under aerobic conditions throughout the experiment. The possibility of anaerobic respiration going on in the depths of the potato cylinder must not be overlooked. Two of the manometers showed a slight positive pressure, which, however, was not indicated by the analyses. In this case, the inability to shake the heavy apparatus and thus oscillate the mercury when taking a reading made it impossible to overcome any adhesion.

The results in this experiment were of interest because they showed the magnitude of the gas exchange. The yield in CO₂, calculated to 0 degree and 760 mm., was 171.3, 171 and 209.1 c.c., respectively. Per gram of potato this corresponded to 7.07, 7.01 and 9.62 c.c.

Similarly, the oxygen consumed amounted to 167, 168.4 and 213.6, respectively which, per gram of potato, represented 6.9, 6.9 and 9.17 c.c. The corresponding values, at 34 C. and 750 mm. pressure, can be obtained by multiplying the figures just given by 1.2036.

It will be seen from table 1 (tubes 1, 4 and 7) that 12-15 gm. of potato removed all of the oxygen in the *h*-tube in less than 46 hours. It should, therefore, be possible to remove all of the oxygen in a jar within 48 hours by introducing sterile potato corresponding by weight to about 15% of the volume. This fact, it will be shown, can be applied to the cultivation of anaerobic bacteria (tables 9-11).

By means of the Novy jar, the real respiratory quotient can be determined with satisfactory precision. It will be seen from the table that the average of the 3 determinations was 0.99. The average for the corrected real respiratory quotient was 1.006.

When working with cultures, a correction is made for the amount of CO_2 dissolved by the medium. Attempts were made to apply the same procedure to the determination of the CO_2 taken up by the potato. There was some uncertainty as to the correctness of the values thus obtained, for the reason that it was quite impossible to break up, at 90 C., the cylinders in the tube through which air was passing. Even after heating for 10 hours, the cylinders were intact.

TABLE 4
RESPIRATORY QUOTIENT OF RAW POTATO, AT 34 C., IN JARS, IN PRESENCE OF EXCESS OF OXYGEN

Experiment No.	1	2	3
No. of days.....	3	5	10
Potato, Gm.	24.2	24.4	22.2
Net air volume.....	2542.35	2018.31	1671.78
Barometer.....	752	752	752
Temperature, C.	34	33.7	33
Observed manometer.....	0	+1.0	+3.0
Calc. real manometer.....	+0.48	-0.36	-3.68
Analyses			
CO ₂	7.973	9.99	14.92
O ₂	13.04	10.93	5.63
N ₂	78.987	79.08	79.45
Corr. analyses *			
CO ₂	7.978	9.985	14.843
O ₂	13.049	10.924	5.601
N ₂	79.04	79.04	79.04
	100.067	99.949	99.484
C c. at 0 degree, 760 mm.			
Dissolved CO ₂	2.82	3.40	2.64
Gaseous CO ₂	168.46	167.63	206.48
Total.....	171.28	171.03	209.12
O ₂ loss.....	167.04	168.48	213.67
Quotients			
Apparent resp.	Average 1.007	0.996	0.973
Real resp.	0.990	0.995	0.966
Corr. real resp.	1.006	1.015	0.979
C c. CO ₂ per 100 Gm. Potato.....	707	701	962

* The initial gas content was assumed to be that of pure air, viz.: CO₂, 0.03; O₂, 20.93; N₂, 79.04.

When boiled in 200 c.c. of 2% H₂SO₄, under a reflux condenser, about 27 hours was needed to hydrolyze 20 gm. of the raw potato cylinders. The slowness of this reaction made the procedure useless for the purpose in mind.

A fairly satisfactory method of determining the amount of CO₂ in the raw potato was eventually obtained. Raw potato cylinders 11 mm. in diameter, and totaling 50-51 gm. in weight, were placed in a liter flask; then 500 c.c.

of distilled water and 10 drops of concentrated H_2SO_4 were added. The flask was closed by a doubly perforated stopper. One opening received the delivery tube through which the washed compressed air was introduced into the liquid; the other one held the lower end of a reflux condenser. The upper end of the condenser led to the $\text{Ba}(\text{OH})_2$ tubes (fig. 15, Part I).

The contents of the flask were raised to boiling and held there for 2 hours. The gas was then shut off, but the air was allowed to pass through the system for about 11 hours. The $\text{Ba}(\text{OH})_2$ tubes were then iced, and the contents titrated as usual, after which they were cleaned, and new baryta solution introduced. The liquid in the flask was decanted, and the potato lumps were transferred to a dish and crushed. The liquid and the potato mash were then recombined, and again heated at the boiling point for 1 hour. After aerating the flask for 1 or 2 hours more, the baryta tubes were titrated as before.

Treated in this manner, 50 gm. of potato gave 11.02 c.c. of CO_2 (0 degree and 760 mm.) in the first distillation and 1.28 c.c. in the second. The total yield, therefore, was 12.30 c.c., or 0.246 c.c. per gm. of potato. Hence, in the calculation of table 4, an allowance of 0.25 c.c. per gm. of potato was made.

Respiration in Pure Oxygen.—In these experiments, the raw potato cylinders were placed in *h*-tubes and attached to manometers. The latter were then connected with the water-pump and with an oxygen tank. After evacuation to 700 mm., oxygen was let in, and the exhaustion, with oxygen refill, was repeated 6 or 7 times. The manometers with the attached tubes were then placed at 34 C. and after 2 hours equilibrated with the tip of cock 3 under water. No control analysis to determine the amount of oxygen present in exper. 1 was made. That for exper. 2 showed 96.8% present. At the conclusion of the experiments, as usual, the potato cylinders in each tube were covered with broth and incubated for 48 hours. The potatoes were sterile, since no growth developed.

The results of 2 experiments of this kind, in triplicate, are given in table 5. It will be noted that a negative pressure promptly developed and reached its maximum of about -50 to -65 in 31-72 hours, depending on the weight and surface of the cylinders. With increase in the percentage or tension of CO_2 ; a corresponding increase in the absorption of this gas by the medium, and especially by the rubber stopper, must take place. The result is a relatively high negative pressure, which is misleading. Since the respiratory quotient, when oxygen is still present, is about 0.99 (table 4), it follows that only a slight negative pressure should exist, provided no absorption of CO_2 occurred.

As in previous experiments, the negative pressure was an expression of the aerobic respiration. When the oxygen disappeared, anaerobic respiration followed, as shown by the fall in the negative pressure. The pressure eventually became positive, the highest reading being $+127$ in 190 hours.

The results of analyses are given in the table. The amount of gas taken for analysis was from 2 to 3 c.c., and consequently some error was unavoidable. This became especially evident in exper. 2, when the nitrogen factor was calculated, and from it the real percentage gain was determined. For tubes 5, 6 and 7 the calculated real gain was 28.1, 34.6 and 24.6, respectively, which would give, for the calculated manometric reading, values unusually higher than the corrected observed readings. For that reason the corrected analyses are not given in the table. The presence of oxygen in tube 5 was accounted for by an error in sampling.

TABLE 5
RESPIRATION OF POTATO IN OXYGEN, *h*-TUBES, AT 34 C.

Experiment No. . . .	I			II			
Tube No.	1	2	3	Control 4	5	6	7
Potato, Gm.	14.3	15.6	15.2	10.5	14.5	11.6	12.0
Hrs.				Hrs.			
Equil., 0.	0	0	0	0	0	0	0
20.	-16	-33	-19	12	-13	-8	-13
24.	21	39	23	36	37	26	35
31.	27	50	31	58	57	50	55
42.	35	65	40	84	-13	-38	63
72.	59	-35	53	108	+10	+2	-14
73.	-58			132	+105	55	+32
92.			-11	156		100	67
98.			+4	189		126	98
116.			+50	190		+127	
				192			+102
Analyses							
CO ₂	88.97	99.40	98.42	0	96.25	97.63	97.44
O ₂	11.03	0.60	0.0	96.81	1.26	0.0	0.0
H ₂	—	—	—	—	0.0	0.0	0.0
N ₂	0.0	0.0	1.58	3.19	2.49	2.37	2.56
	100.00	100.00	100.00	100.00	100.00	100.00	100.00

From the corrected observed manometric readings which, unless much CO₂ is taken up by the rubber stopper, approximate the truth, it is possible to deduce the probable nitrogen factor and from this the percentage of real gain.

$$\text{Since the pressure or manometric reading} = \frac{G \text{ or } L \times (B-T)}{100}$$

$$\text{it follows that the gain or loss in \%} = \frac{100 \times \text{Man. Pr.}}{B - T}$$

$$\text{And, the N}_2 \text{ factor} = \frac{100 + \text{Gain or } - \text{Loss}}{100}$$

From the N₂ factor thus obtained, the corrected values for CO₂ present in tubes 5, 6 and 7 were estimated to be 110.7, 115.6 and 111.7%, respectively. Since rubber stoppers were used in the tubes, it is certain that an appreciable amount of CO₂ was taken up by the rubber. This, in fact, is the explanation of the high negative pressure observed. From these experiments, it follows

that, in an *h*-tube having a capacity of about 100 c.c. and filled with pure O₂, the raw potato (11-15 gm.) can remove all of the oxygen in about 72 hours. The respiratory quotients in exper. 2 would be greater than 1 because of the secondary anaerobic respiration.

Respiration in Pure Nitrogen.—It has been shown in the preceding experiments that anaerobic respiration followed the aerobic as soon as the oxygen was greatly reduced or completely removed. It was desirable to test the anaerobic respiration without previous exposure to oxygen, except such as was necessary in preparing the cylinders. It is well known that the anaerobic bacteria produce H₂ as well as CO₂, while the yeast under like conditions makes CO₂ and alcohol. In all of the work on the respiration of the raw potato in air, no direct tests were made for hydrogen. The manometric readings, calculated and observed, usually agreed sufficiently to exclude the presence of this gas. Tests for hydrogen and hydrocarbons (marsh-gas), however, were made in 3 of the tests with pure oxygen, and were negative. It will be seen from the following experiments with the potato in pure nitrogen that hydrogen or other combustible gas was also absent. It follows therefore that the anaerobic respiration of the potato is unlike that of the ordinary anaerobic bacteria. The question of alcohol production will be taken up presently.

The results of 2 experiments with nitrogen are given in table 6. The method of procedure here was the same as in the experiments with oxygen, except that the tubes were filled with pure nitrogen. At the conclusion of each test, broth was added to the tube, which was then incubated for 48 hours or more. In every case, the potatoes were thus proved to be sterile.

Table 6 shows that a positive pressure rapidly developed. In 2 days, it approximated +100, and in tube 2 it reached +162 mm. in 86 hours.

The control for exper. 2 served to show the purity of the nitrogen atmosphere, and was used in recalculating the analytical data to the nitrogen basis. In exper. 1, no control tube was made, but since the tubes were evacuated and refilled with nitrogen 10 times it was assumed that they contained 100% of nitrogen at the start.

The corrected analyses showed a maximal yield of nearly 24% of CO₂. It is possible that with a longer period of incubation this amount could have been increased. No attempt, however, was made to determine the time at which anaerobic respiration ceased. Likewise no attempt was made to evacuate and refill the tubes with pure nitrogen in order to determine whether the anaerobic respiration would be resumed with its original vigor.

Production of Alcohol.—The question of alcohol production by plant tissue is not a new one, as will be pointed out in the discussion. It appeared desirable, however, to ascertain whether or not alcohol was made during the anaerobic respiration of the raw potato cylinders. Accordingly, the following experiment was planned with this object in view.

Each of 10 tubes, 20 x 150 mm., received 3 potato cylinders. The total weight of the cylinders was 133.2 gm. The cotton plugs were then cut, flamed

TABLE 6
RESPIRATION OF POTATO IN NITROGEN, *k*-TUBES, AT 34 C.

Experiment No.	I		II				
Tube No.	1	2		Control 4	5	6	7
Potato, Gm.	15.2	14.1		12.5	15.1	12.5	13.3
Hrs.			Hrs.				
Equil., 0.....	0	0	0	0	0	0	0
11.....	+29	+34	12	+25	+18	+23
34.....	83	71	36	84	66	58
39.....	92	82	58	+107	97	84
46.....	+109	88	84	118	106
57.....	117	108	127	125
86.....	...	+162	112	+130
			132	138
			156	148
			163	+148
Corr. obs. man.	110.7	164.1	108.7	132.9	150.0
Calc. real man.	122.7	169.1	124.2	146.3	164.4
Analyses							
CO ₂	14.15	19.28	0.0	15.56	17.54	19.29
O ₂	0.0	0.10	0.48	0.09	0.0	0.0
H ₂	—	—	—	0.0	0.0	0.0
N ₂	85.85	80.62	99.52	84.35	82.46	80.71
	100.00	100.00	100.00	100.00	100.00	100.00
Corrected analyses							
CO ₂	16.48	23.92	18.36	21.17	23.78
O ₂	0.0	0.12	0.10	0.0	0.0
H ₂	—	—	0.0	0.0	0.0
N ₂	100.00	100.00	99.52	99.52	99.52
	116.48	124.04			117.98	120.69	123.30

and pushed within the tubes. These were then placed upright in a tall Novy jar, of about 3,300 c.c. capacity, which was then sealed in the usual way. The jar was then evacuated to —400 mm. and refilled with nitrogen. This operation was repeated 6 times. A sample of the gas was then drawn, and analysis showed that it contained 0.93% of O₂. The jar was then placed in the hot-room at 34 C. for 29 days. The potatoes showed no change in appearance.

It was then analyzed and found to contain 9.92% of CO₂ and no oxygen. Recalculated to the nitrogen basis, this gave 10.85%, from which data a pressure of +66.6 mm. was deduced.

The net volume of the jar was 2,936 c.c., and hence the yield of CO₂ under anaerobic conditions, unreduced, was 318.5 c.c., or 2.4 c.c. per gm. of potato. Since a manometer was not attached to this jar, it was not possible to determine the exact volume of CO₂ at 0 degree and 760 mm. Assuming the

reduced volume to be 250 c.c., this would correspond to 0.494 gm. of CO_2 . If alcohol were formed, as in alcoholic fermentation, it should amount to 0.539 gm.

In order to detect the presence of alcohol, the potato cylinders were transferred to a large flask, and the inside of each tube was washed out with distilled water. The washings were added to the flask, and enough water was added to make up 1 liter. The whole was then distilled until about 750 c.c. had passed over. The potato cylinders were then mashed, another liter of water was added, after which about 900 c.c. were distilled off.

The two distillates were then combined and redistilled, yielding 1,100 c.c. The second distillate was acidified with H_2SO_4 and again distilled so as to give 630 c.c. The latter was again acidified and redistilled, thus giving 400 c.c. This was now rendered alkaline with Na_2CO_3 and again subjected to distillation, yielding 280 c.c. The latter was acidified once more and concentrated as before to 190 c.c. The 7th distillation reduced the volume to 125 c.c. and the 8th to 85 c.c.

A pycnometer with a capacity of 50 c.c. and provided with a thermometer was used to determine the density. The weights obtained, corrected for the temperature ($20\frac{1}{4}$ C.) gave a density of 0.99780, which corresponded to 0.22 gm. of alcohol for 100 c.c. The amount recovered in the 8 successive distillations was therefore 0.187 gm., or about one-third of the quantity indicated by the CO_2 findings.

The distillate gave a distinct iodoform test, but no further identification was attempted. This experiment, therefore, showed the probable presence of alcohol. To be decisive, it would have to be carried out on a larger scale. Likewise control tests, subjecting fresh potato to distillation, would have to be made.

Respiration of Immersed Potato.—The preceding work has shown that the sterile raw potato rapidly consumed the overhead oxygen and produced CO_2 , with the result that the respiratory quotient, under aerobic conditions, approximated 1. In view of the fact that the raw potato favors the growth of anaerobic bacteria, it was desirable to ascertain the extent of the gas exchange when the potato was immersed in a fluid. It was to be expected that the dissolved oxygen would be consumed and that the anaerobic respiration would then manifest itself. The latter respiration is essentially a proof that free oxygen is absent.

In these experiments the sterile potato, either raw or autoclaved at 120 C. for 15 minutes, was completely covered with the sterile fluid. The water or broth was autoclaved in a globe funnel the end of which was provided with a drawn-out tube, thus facilitating transfer of the sterile fluid. The *h*-tube was used in all of these tests. The tubes, with the attached manometers, were placed in the hot-room at 34 C., and were equilibrated after 2 hours. The sterility of the content of a tube was shown by the absence of any cloudiness in the broth. In 6 experiments, in which 39 tubes were used, contamination occurred but 3 times.

An essential condition in these tests is that the potato shall be wholly immersed in the fluid. To accomplish this the manometers were inclined as far as possible but, even then, it was noted that the depth of the fluid over the potato varied. It is possible that a thin layer of liquid will bring more dissolved oxygen into contact with the potato than a deep one. The exposure of even a small piece of the potato radically changes the result.

The first experiment was made with sterile distilled water, raw and autoclaved potato being used. The results are given in table 7. It will be noted that the tubes with raw potato had a slight negative manometric phase, which rapidly gave way to a positive pressure indicative of anaerobic change. The analyses showed a slow decrease in oxygen and a more marked increase in CO_2 production. The respiratory quotients, which ranged from 1.5 to 3.6, confirmed the presence of anaerobic respiration.

By contrast, the autoclaved potato showed practically no respiratory change. This, of course, could be expected in view of the behavior of such potato in air (table 2). The slight increase in the negative pressure was due, in part, to oxidative changes. In 15 different tests with immersed, autoclaved potatoes, only 4 gave a slightly higher negative pressure (7.8, 9, 10 mm.), and in these a part of the potato was exposed. In 7 tubes, the pressure varied from 0 to -4 . The indicated respiratory quotient was, as a rule, less than 0.5 and in reality was probably much less. The manometric results with autoclaved potatoes were essentially the same whether immersed in water, plain broth, glucose broth or glycerol broth.

Similar experiments to the one just given were made with the raw potato immersed in plain broth, 2% glucose broth or 5% glycerol broth. The same minimal negative phase was observed in the tests with plain or glycerol broth. In glucose broth, however, a positive pressure developed from the start. The positive pressure rapidly rose and with plain broth and glucose broth, in one experiment, it reached $+160$ mm. in about 130 hours. The potato with glycerol broth invariably gave lower readings than did potatoes with plain or glucose broth. It would seem that the viscosity of the liquid hindered the solution of oxygen. On the other hand, it is possible, as pointed out below, that activity of the enzymes is partially inhibited by the glycerol.

One experiment in which the 3 broths were used at the same time is given in table 8. The results with the 6 tests there given were not materially different from 15 other trials. It will be seen that the oxygen removal was slow, particularly so with glycerol broth. The CO_2

production was considerably higher than the oxygen loss, and, as a result, the real respiratory quotients were high. As given in the table, they range from 4.3 to 7.6.

The analyses of the glycerol tubes showed less CO_2 production and less oxygen consumption than was the case with the other tubes. This fact would indicate that, to some extent, the glycerol inhibited the action of the respiratory enzymes.

On the assumption of a zymase-like action of the active potato constituent, it was expected that the results with glucose broth would

TABLE 7
RESPIRATION OF POTATO, RAW AND AUTOCLAVED, IMMERSSED IN STERILE WATER, AT 34 C.

	Raw			Autoclaved		
Tube No.	1	2	3	4	5	6
Potato, Gm.	9.4	12.9	11.7	9.4	8.9	8.8
Hrs.						
Equil., 0.	0	0	0	0	0	0
1.	-1	-1	-1	-1	-2	-1
12.	-5	+3	+1	2	3	3
23.	13	8	2	5	3	4
35.	22	13	8	6	3	5
40.	+26
48.	25	19	-6	3	5
69.	+32	-6	..
84.	+32	-6
Corr. obs. man.	26.3	32.3	32.6	-6.1	-6.1	-6.1
Calc. real man.	36.5	32.5	23.6	-0.8	-4.5	-2.9
Analyses						
CO ₂	6.86	9.06	9.31	0.50	0.24	0.59
O ₂	18.01	15.39	14.21	20.37	20.21	20.04
N ₂	75.13	75.55	76.48	79.13	79.55	79.37
	100.00	100.00	100.00	100.00	100.00	100.00
Corrected analyses						
CO ₂	7.217	9.478	9.622	0.499	0.239	0.587
O ₂	18.947	16.101	14.685	20.347	20.080	19.957
N ₂	79.04	79.04	79.04	79.04	79.04	79.04
	105.204	104.619	103.347	99.886	99.359	99.584
Real resp. quot.	3.638	1.958	1.536	0.801	0.237	0.568

greatly exceed those given by other mediums. This, however, was not the case. Plain broth appeared to give equally good results, and this would seem to indicate that the anaerobic CO_2 production was not associated with a zymase-like body. However, further tests are required to determine this point. The respiratory quotients with either plain or glucose broth may rise to 20 and even above 50, depending on the time element and the amount of potato, if not its surface area.

It can be concluded from these experiments that the immersed raw potato can and does absorb the dissolved oxygen which is present in

the broth. Further, that by so doing it renders the medium suitable for the growth of anaerobic bacteria. On the other hand, the autoclaved potato possesses little or no action of this kind. Some slight auto-oxidation of the heated potato may account for such results as have been obtained with anaerobes.

THE POTATO AND GROWTH OF ANAEROBES

The preceding work demonstrated (1) that the raw potato carries on an active respiration which results in the complete removal of the

TABLE 8
RESPIRATION OF RAW POTATO, IMMERSSED IN BROTHS, AT 34 C.

	Plain		Glucose		Glycerin	
Tube No.	1	2	5	6	7	8
Potato, Gm.	7.1	6.7	7.4	7.0	7.7	8.2
Hrs.						
Equil., 0.	0	0	0	0	0	0
2.	-1	-1	+2	+2	+1	0
5.	+1	0	5	5	2	0
19.	13	13	15	13	8	+2
43.	36	35	31	28	26	14
48.	+42	..	+35	..	+31	..
69.	63	..	50	..	18
91.	92	..	75	..	34
95.	+88	+38
116.	+131
Corr. obs. man.	43.0	90.43	35.6	133.1	31.5	38.4
Calc. real man.	44.6	88.1	34.4	132.9	28.5	42.2
Analyses						
CO ₂	7.27	13.21	5.76	18.22	4.98	7.34
O ₂	18.38	16.50	18.87	15.24	19.04	18.07
N ₂	74.35	70.29	75.37	66.54	75.98	74.59
	100.00	100.00	100.00	100.00	100.00	100.00
Corrected analyses						
CO ₂	7.729	14.854	6.040	21.643	5.181	7.778
O ₂	19.539	18.554	19.789	18.103	19.806	19.148
N ₂	79.04	79.04	79.04	79.04	79.04	79.04
	106.308	112.448	104.869	118.786	104.027	105.966
Real resp. quot.	5.567	6.161	5.305	7.669	4.615	4.367

oxygen present in a tube; and (2) that the immersed potato rapidly creates anaerobic conditions in the liquid, as shown by the high respiratory quotients. These facts in themselves offer a clear explanation of the rôle that the potato plays when it is added to a broth inoculated with an anaerobic organism. The latter organism is enabled to grow because the potato has removed all of the dissolved oxygen.

It becomes in nowise necessary to resort to the theory of a catalase destroying an hypothetical peroxide. The respiratory demand of the

living raw potato is the simple factor involved in the reaction. Plausible as this assertion may be, it does not take into account the possibility that some additional condition, some growth-promoting factor for example, is supplied to the broth by the potato. It was precisely to exclude the latter possibility that a series of cultural experiments were made with "separated" potato and inoculated broth. If the potato, which is not in contact with the inoculated broth, exerts its action at a distance, then it would be a crucial test showing that oxygen removal was the sole and only factor involved.

Broth Culture with Distant Potato.—In these experiments, *h*-tubes were employed. About 10 c.c. of sterile glucose broth were placed in the side-arm of each tube, and then inoculated with a drop of a culture in glucose litmus gelatin, rich in spores. Into the main arm of the even numbered tubes, sterile raw potato cylinders were then introduced. The tubes without potato served as controls. The cotton plugs were then trimmed, flamed and pushed into the tubes, each of which was then connected with a rubber stopper to a manometer. The latter, with the attached tube, was then placed in the hot-room, at 34 C., and after 2 hours it was equilibrated.

The results obtained in one experiment with the organisms of symptomatic anthrax, botulinus and malignant edema are given in table 9. It will be noted that control tubes 1, 3 and 5 showed practically no manometric change, no change in the air content and no growth.

The potato tubes, on the other hand, gave the usual manometric response. The crest of the negative phase was passed in 18-24 hours, and the readings became strongly positive in 65 hours. The analyses made at that time showed the complete absence of oxygen and the presence of 30% or more of CO₂.

In the broth of these tubes, cloudiness was first observed in 41 hours. The growth was then slight but distinct. In the next 24 hours, it increased considerably. Microscopic examination revealed rich cultures, with typical, actively motile rods, and many spores.

Similar experiments were made with 3 other anaerobes, namely *B. novyi*, *B. tetani* and *V. septique*. Three separate tests made with the first of these gave a slight to moderate cloudiness or growth, with some gas production in one instance. The relative failure of this organism was probably due to the fact that the culture used was very poor in spores. The bacillus of tetanus was likewise tested 3 times, the growth being slight in 2 trials, but in the 3rd it was rich with abundant spore production. *V. septique* presented no difficulty, yielding a heavy growth with much gas. Even the control tube (no. 5) in

table 10 gave a slight cloudiness on account of the large volume of broth (20 c.c.) which was used for that test. Attention may be called to the fact that the analysis of this tube showed that some oxygen consumption took place. As the test was not repeated, it is not possible to draw the definite conclusion that some oxygen utilization occurred with *V. septique*. Oxygen will necessarily be taken up if reduction products are present.

An examination of table 10 will reveal the same features that have been brought out in connection with the preceding table. The potato tubes showed positive growths and an entire absence of oxygen, while the control tubes were negative as to growth, with the exception of

TABLE 9
BROTH CULTURES OF ANAEROBES, WITH DISTANT POTATO,, *h*-TUBES, AT 34 C.

Tube No.	B. chauvoei		B. botulinus		B. oedematis	
	1c	2	3c	4	5c	6
Potato, Gm.	0	14	0	15.6	0	18.7
Hrs.						
Equil., 0.....	0	0	0	0	0	0
2.....	0	-3	0	-5	0	-3
15.....	0	17	0	13	0	18
18.....	0	16	+1	17	+1	23
24.....	0	-20	2	-8	+1	-17
41.....	0	+7*	2	+28*	0	+16*
63.....	+1	85	2	99	0	103
65.....	..	+97	..	+110	..	+114
92.....	+1	..	+2	..	-4	..
Growth.....	0	++	0	++	0	++
Analyses						
CO ₂	0.55	30.10	0.29	30.87	0.48	31.09
O ₂	20.04	0.0	20.22	0.0	20.31	0.0

* Cloudiness first observed.

tube 5 in which apparently a slight development was initiated but in the end was inhibited by the excess of oxygen.

It is evident from these experiments that the raw potato can work at a distance from the inoculated broth. It favors the growth, therefore, by creating an oxygen-free atmosphere which necessarily brings about the disappearance of the dissolved oxygen. The growth of the anaerobic organism is promoted by raw potato regardless of whether this is immersed in the broth or is at a distance from it. In the absence of analyses, either condition suggests something mysterious.

Plate Culture with Distant Potato.—Since, as has been shown above, it is possible to obtain cultures of anaerobes in broth with distant

potato, it was evident that similar results could be expected with plate cultures. The essential condition would be to have sufficient sterile potato in the anaerobe jar so that the oxygen would be quickly removed. It was clear, however, that the sterility of the potato cylinders could be maintained by placing them in sterile test-tubes, and the number of these would be such as leave little or no space for the plates. Accordingly, the idea of using sterile potatoes was given up.

It seemed, however, that essentially the same result could be obtained by the use of raw potato with no attempt at sterility. Under such conditions, the respiration of the potato would be supplemented by that

TABLE 10
BROTH CULTURES OF ANAEROBES, WITH DISTANT POTATO, *h*-TUBES, AT 34 C.

Tube No.	B. Novyi		B. tetani		V. septique	
	1c	2	3c	4	5c	6
Potato, Gm.	0	14.4	0	19.5	0	16.4
Hrs.						
Equil., 0.....	0	0	0	0	0	0
3.....	+1	-10
12.....	0	-21	+4	-22
18.....	0	38
23.....	0	29
29.....	0	-5
36.....	+5	+8	5	+9
41.....	+1	+87*
45.....	4	114
49.....	7	+147
60.....	5	52*	6	77*	13*	..
84.....	+5	92	+7	130
88.....	..	+98	..	+140	14	..
117.....	+14	..
Growth.....	0	+	0	++	?	++
Analyses						
CO ₂	0.19	31.09	0.68	28.02	3.06	27.75
O ₂	20.87	0.0	20.26	0.0	18.36	0.0

* Cloudiness first observed.

of the aerobic organisms present, and, with the removal of the oxygen, growth of anaerobes should occur on the plates. This proved to be the case. Anaerobiosis can be produced in a jar by the simple procedure which will be described. Without doubt, the method can be used to advantage when hydrogen is not available. In such case, it is advisable to place in the jar a Petri dish with about 10 c.c. of 5-10% solution of KOH in order to reduce the CO₂ content.

The raw potato was peeled and then cut into thin slices, which in turn were cut into narrow strips. These were placed in water, where they were kept until the jar was ready for use. They were then roughly dried by pressing between layers of muslin, and 250-300 gm. were placed on the bottom of Novy jars of about 2,000 c.c. capacity. A tripod, made of glass rods, was

then put in the jar, and on top of this the plates (4-6) were set. The jar was then sealed in the usual way (Part I) and placed at 34 C. for 4-7 days. Since considerable pressure develops in the jar, the main stopper should be securely wired and wedged. During the first 2 or 3 days, the potato strips showed no change, but after that time, because of bacterial action, they gave off water, and gas formation took place. In about a week, the potato pieces were completely dissolved.

Each of 6 anaerobes was tested for its growth on the surface of glucose agar plates, as well as for the production of colonies, in poured plates. The material for seeding was usually obtained from glucose litmus gelatin tubes. The deposit was taken up in a Pasteur pipet, and one drop was placed on the middle of a solidified agar plate, and the liquid was carefully spread over the surface by means of a bent rod. Another drop was added to agar at 50 C., and this was then poured into a plate; 12-15 c.c. of agar were used for each plate.

The results of these experiments, together with like tests with distant *B. subtilis*, which will be described later, are given in table 11. The

TABLE 11
PLATE CULTURES OF ANAEROBES WITH DISTANT POTATO, AND *B. SUBTILIS*, AT 34 C.

Growth.....	Potato		<i>B. subtilis</i>	
	Colonies	Surface	Colonies	Surface
<i>B. botulinus</i>	++	++	++	++
<i>B. oedematis</i>	++	++	++	++
<i>B. chauvoei</i>	++	++	++	++
<i>B. tetani</i>	++	++	0	0
<i>B. Novyi</i> , exp. 1.....	0	0	+	+
<i>B. Novyi</i> , exp. 2.....	0	0	0	0
<i>B. Novyi</i> , exp. 3.....	+	+
<i>V. septique</i> , exp. 1.....	0	0	+	+
<i>V. septique</i> , exp. 2.....	0	0	0	0
<i>V. septique</i> , exp. 3.....	+	+

first 4 organisms gave excellent colonies in 7 days, at which time the jars were analyzed and opened. The colonies were well developed on the 4th day.

The surface growth of these organisms on the streaked plates was equally marked. Examined under a no. 7 objective, after a cover-glass had been placed on the plate, it showed masses of typical bacilli with an abundance of spores.

The results were not as favorable with *B. novyi* and *V. septique*. This was in part due to the scarcity of spores in the inoculum. Clearly positive results were obtained with these 2 organisms only on the 3rd trial, which extended over 33 days. It is likely that the high content of CO₂ was an inhibitive factor.

Samples of gas were withdrawn from the 7 jars used in these experiments, and analyzed. They contained no oxygen, and from 55 to 75% of CO₂. No test was made for the presence of combustible gases.

In these experiments, the development of colonies in the thin layer of agar, and particularly the growth on the surface, was clearly a consequence of the total removal of oxygen brought about by the distant action of the potato and the associated aerobes.

MICROBIC ASSOCIATION

It has been pointed out that the failure to obtain a "getrennte" symbiosis led von Oettingen to reject Pasteur's interpretation of the part played by aerobes in the so-called "mixed cultures." Had he resorted to the analysis of the air content of his culture tubes, he would have discovered his error in assuming that all of the oxygen had been removed. An aerobe in broth culture does not consume as much oxygen as it does when growing on the surface of slanted agar. In the liquid, the anaerobic condition is soon established, and a strict aerobe can obtain a further supply of oxygen only by growing on the surface. The work on the respiration of potato in air, contrasted with that when immersed in broth, illustrates this point. That growth of anaerobes can be obtained by the use of a distant culture of an aerobe will be seen from the work which follows.

Broth Culture with Distant B. subtilis.—As in the similar experiments with potato, *h*-tubes were employed. The side-arms of each received 10 c.c. of glucose broth, while the main arms were given 10 c.c. of glucose agar. The latter medium was used because the Hay bacillus when grown on it removes the oxygen more rapidly than when cultivated on plain agar. After sterilization in the autoclave, the tubes were slanted. The broth in each of a pair of tubes was inoculated with 2-3 drops of the anaerobe culture to be tested. The entire agar surface of the even numbered tubes was then inoculated with *B. subtilis*. The odd numbers received no Hay bacillus and served as controls. The tubes were then attached to manometers in the usual way, placed in the hot-room and equilibrated after 2 hours.

The results of these tests are given in tables 12 and 13. Positive and rich growths were obtained with *B. chauvœi*, *B. botulinus* and *B. tetani*. The growth was less abundant in the case of *V. septique*. All of these showed an abundance of spores. On analysis, no oxygen was found in these tubes.

No growth was obtained in this experiment with *B. novyi*, but analysis showed the presence of 0.19% of oxygen. In another test with this organism, the manometer gave a reading, in 132 hours, of only + 5, but on analysis no oxygen was present, while the CO₂ was 20.97%. Growth also failed in this tube.

The bacillus of malignant edema likewise failed to grow in this and in a subsequent trial. In both cases, however, oxygen absorption

was incomplete, 6.9 and 3.1% of oxygen being found. The failure to obtain growth in the case of the two organisms mentioned was probably due to the rather slow and even incomplete removal of oxygen by the Hay bacillus. The growth of the latter was not as rich as it should have been.

The control tubes showed no growth, and on analysis 4 out of the 6 tubes exhibited little change in the composition of the air, as was to be expected. The other 2 tubes (nos. 3 and 5) showed, with apparently

TABLE 12
BROTH CULTURES OF ANAEROBES, WITH DISTANT *B. subtilis*, *h*-TUBES, AT 34 C.

Tube No.	<i>B. chauvoei</i>		<i>B. botulinus</i>		<i>B. oedematis</i>	
	1†	2	3†	4	5†	6
Hrs.						
Equil., 0.	0	0	0	0	0	0
1.....	0	-2	0	0	0	-2
12.....	0	+3	0	+2	0	-2
36.....	0	4	0	3	0	+7
61.....	-1	39*	-1	3	0	34
83.....	2	67	2	37*	-1	34
88.....	..	+75	+85
109.....	3	..	2	104	2	..
137.....	3	..	4	+142	2	..
140.....	-3	..	-4	..	-5	..
Growth.....	0	++	0	++	0	0
Analyses						
CO ₂	0.48	27.57	9.24	20.49	9.41	18.08
O ₂	19.72	0.0	10.91	0.0	11.01	6.91

* Cloudiness first observed.

† Control with no *B. subtilis*.

no reason, considerable reduction of oxygen. In a duplicate test with the bacillus of malignant edema, this removal of oxygen by the control was absent.

Plate Cultures with Distant B. subtilis.—Given a sufficient area of *B. subtilis*, it is possible to remove completely the oxygen content in a Novy jar, and thus allow anaerobes to grow. This was actually done several years ago by Dr. Roehm while working in this laboratory. As his results were unpublished, it was desirable to repeat the work with additional organisms.

As in the case of the corresponding experiments with raw potato, each of 6 anaerobes was tested for its growth on the surface of glucose agar plates, as well as for the production of colonies in poured plates. The method of procedure in preparing these plates was the same as given in connection with table 11.

In one experiment, the tall form of Novy jar was used because it could hold the entire set of 18 plates. The surface of 6 of these plates was inoculated with *B. subtilis*, care being taken to spread the inoculum all over the agar. Two of these plates were placed on the bottom, and then above these the others were set, 3 plates of anaerobes alternating with one of the aerobe.

The air capacity of the jar when empty was 3,321 c.c. It was then sealed and placed in the hot-room, at 34 C., for 33 days, when it was analyzed and found to contain 27.01% CO₂ and no oxygen.

In a second trial, a jar of about 2,000 c.c. capacity received 4 plates of the Hay bacillus and 2 plates each of *B. novyi* and *V. septique*. At the end of 7 days, when analyzed, it had 21.45% CO₂ and no oxygen. In this test, there was no evidence of growth of the former, and the result with the latter organism was doubtful.

When jars are kept in the hot-room for a week or more, considerable desiccation of the agar plates may take place as a result of distillation. Hence, it is best to use at least 15 c.c. of agar on a plate.

TABLE 13
BROTH CULTURES OF ANAEROBES, WITH DISTANT *B. SUBTILIS*, *h*-TUBES, AT 34 C.

Tube No.	<i>B. Novyi</i>		<i>B. tetani</i>		<i>V. septique</i>	
	7†	8	9†	10	11†	12
Hrs.						
Equil., 0.....	0	0	0	0	0	0
1.....	-1	0	-1	-2	0	0
12.....	1	-3	2	2	0	-3
36.....	1	2	3	6	-1	1
61.....	1	2	8	-1	1	-3
83.....	2	3	11	+14*	2	+57*
109.....	2	2	14	21	2	83
137.....	2	2	15	+22	2	+85
140.....	-16	..	-3	..
156.....	-3	-5
Growth.....	0	0	0	++	0	+
Analyses						
CO ₂	0.38	19.90	0.68	21.65	0.39	23.16
O ₂	17.47	0.19	19.22	0.0	20.14	0.0

* Cloudiness first observed.

† Control with no *B. subtilis*.

The results of these experiments, together with those obtained with distant potato, are given in table 11. In the first experiment with 12 anaerobe plates in one jar, only the 2 plates of *B. tetani* failed to develop. This was the more striking since in the companion experiment with the distant potato the tetanus bacillus grew very well. The plates of *B. novyi* were rather poor and not as good as those in the potato jar. The other 4 anaerobes gave numerous typical colonies on the poured plates, while on the surface plates good growths were present, with abundant spores.

Without doubt, these experiments with distant Hay bacillus could be extended so as to obtain positive results with every one of the anaerobes tested. The results, such as they are, clearly demonstrate that the mode of action of an aerobe in "mixed culture" is that of an oxygen remover. In the "mixed culture," it is relatively easy for the aerobe to remove the small amount of dissolved oxygen, and hence the anaerobe develops quickly and well. With a distant culture, the removal

of the oxygen is a much slower process, and consequently some of the anaerobes may be injured, if not actually killed.

Mixed Cultures with Pneumococcus.—McLeod and Gordon, and also Callow⁶⁶ expressed the view that anaerobes could not grow in air because they made H_2O_2 and did not produce a protective catalase. The formation of H_2O_2 was hypothetical, but the absence of catalase was demonstrated. However, the addition of catalase from bacteria, fat, yeast (Callow) and from liver failed to promote growth. It seemed that the theory of H_2O_2 production and inhibition could be tested by means of "mixed" cultures with aerobes which are said to produce H_2O_2 and little or no catalase. The group of peroxide producers is stated to include many streptococci, lactic acid bacilli, certain sarcines and pneumococci. More than 40 different aerobes have been tried out by different observers and found to promote the growth of anaerobes in so-called "mixed cultures." Among these are to be found streptococci, sarcines and lactic acid bacilli, but apparently the pneumococcus has not been used. In view of the recognized production of H_2O_2 by the pneumococcus, it was desirable to ascertain whether or not this organism would favor the growth of anaerobes in the presence of air.

The experiment was made by inoculating 6 tubes of glucose broth with pneumococcus I. The tubes were placed over night at 34 C. to secure a good growth. They were then seeded, each with one of the 6 anaerobes used in the preceding tests. At the same time, as controls, 6 other tubes of glucose broth were inoculated with the anaerobes. The inoculated tubes and the controls were then placed at 34 C.

Because of absence, the tubes were not examined until the 7th day. At that time the "mixed cultures" showed a slight to a heavy deposit of growth, while the controls had practically none. On the 23rd day, the tubes were examined microscopically, and showed that growth of the anaerobes had taken place with varying degree of spore formation. The slight sediment in the control tubes contained nothing more than the detritus from the inoculated material.

This experiment therefore demonstrated that the pneumococcus, like all other known organisms, favored the growth of anaerobes in air. Since it is considered to have no catalase, the favoring action cannot be due to the destruction of the hypothetical peroxide. Moreover, though looked upon as a good peroxide producer, it is not capable of producing enough of it under these conditions to prevent the growth of the anaerobes. It follows therefore that the observed growth must be ascribed to the removal of the dissolved oxygen by the pneumococcus.

⁶⁶ Jour. Path. & Bacteriol., 1923, 26, pp. 320-325.

DISCUSSION

The respiration of plants, more especially of leaves, seeds, sprouts, roots, tubers, flowers and fruit, has been studied by various workers. It is beyond the scope of this paper to discuss either the methods used or the results obtained. It is recognized that CO_2 is given off by plants under aerobic and anaerobic conditions. The existence of anaerobic respiration led to the assumption that the process involved was similar to that of alcoholic fermentation by the yeast plant. The first proof of this was given by Godlewski (1897). It was developed more fully by Stoklasa (1903), who endeavored to show that the anaerobic respiration of plant tissue (and also of animal tissue) under presumably sterile conditions resulted from changes that were essentially of the type of alcoholic fermentation. Some confirmation of this view as regards plants was presented by Palladin and Kostytschew⁶⁷ (1906).

Of direct interest are the few experiments which Stoklasa⁶⁸ made with whole potatoes, kept under water, through which a current of hydrogen was passed. Approximately equal amounts of CO_2 and alcohol were obtained. From his 3 experiments, extending over 7 days, at 22-24 C., it can be calculated that 109 gm. of potato yielded 1.3 gm. of alcohol and 1.28 gm. of CO_2 . The latter corresponds to 650 c.c. at 0 degree, 760 mm. A kilogram of fresh potato gave only 14 mg. of alcohol.

It can be calculated from the experiments given in table 4 that 100 gm. of potato, under aerobic conditions, yields 701-962 c.c. of CO_2 at 0 degree, 760 mm. Similarly, from tube 7, table 6, in which the potato cylinder was kept in nitrogen, for 6 days, it can be computed that 100 gm. would have yielded about 216.5 c.c. of CO_2 , unreduced. In the experiment which was intended to show the production of alcohol, the yield of CO_2 , in 29 days corresponded to 240 c.c. per 100 gm. of potato. The amount of alcohol supposedly recovered in that experiment was 0.187 gm., or 0.14 gm. per 100 gm. of potato, which is only about one-tenth that obtained by Stoklasa with the whole potato.

This study of sterile raw potato cylinders leaves no room for doubt that active aerobic respiration is maintained as long as there is a free supply of oxygen. The respiratory quotients under these conditions

⁶⁷ Ztschr. f. physiol. Chem., 1906, 48, pp. 214-239.

⁶⁸ Footnote 34, p. 309.

approximate 1, the value which would be expected for the oxidation of carbohydrates. It is not to be assumed that the whole potato is capable of an equally intense aerobic respiration. Rather it is to be expected that the skin would tend to restrict the oxygen contact and hence would favor the anaerobic type of respiration.

The aerobic respiration of the potato cylinder is of special significance in that it affords a simple, rational explanation of the way in which the potato favors the growth of anaerobes. Immersed in water, it quickly takes up the dissolved oxygen and further oxidative changes of this type cease. The well-known practice of placing cut potatoes in water, in order to retain the original whiteness, has its basis in the fact mentioned.

The immersed potato, having exhausted the dissolved oxygen, promptly begins to respire as an anaerobe, which fact is evidenced by the rise in the respiratory quotient. The CO_2 which is thus produced cannot be accounted for by assuming the production of acid products which react with the carbonate normally present. The respiratory process, whether aerobic or anaerobic, is strictly a vital one in the sense that one or more enzymes are concerned.

There is no need for the assumption that the potato cylinder when immersed in broth favors the growth of anaerobes through the action of its catalase in destroying the hypothetical H_2O_2 . As crucial evidence against this view is the fact that distant potato promotes the growth of anaerobes as well as one that is immersed. It eliminates the theory of peroxide formation, and likewise the assumed catalase action. The distant action clearly consists in the removal of the atmospheric oxygen. As the tension of the oxygen in the air decreases, the dissolved oxygen, held by the medium, is liberated, and in the end an oxygen-free environment is established. It is then that the anaerobe can multiply whether in broth, in agar or on the surface of agar plates.

Apart from the theories of Kedrowski, von Oettingen and Tarozzi, there is perhaps a general acceptance of the view held by Pasteur that in microbic association the result is due to the removal of the dissolved oxygen by the aerobe. Positive proof of this, if such is needed, is supplied by distant cultures with the Hay bacillus. Here, as in the case of the potato, aerobic respiration results in the removal of all of the oxygen, and hence the anaerobe is enabled to grow in distant broth, in plates, or even on the surface of agar. As stated before, the so-called aerobic growth of anaerobes is but an illusion which rests on the erroneous assumption of the presence of oxygen. The fact that there

is air in the culture tube does not necessarily mean that the medium contains dissolved oxygen.

The question as to why anaerobes cannot grow in the presence of air has not been touched on. It is intimately connected with another question, namely, why aerobes cannot grow in the absence of oxygen. It can be surmised that two different types of enzymes are concerned in aerobic and anaerobic respiration, and, further, it can be assumed that both kinds are present in the potato and in facultative anaerobes, since these show both types of respiration. The obligative aerobe would therefore be provided with but one tool, the one which is activated by free oxygen. Similarly, the obligative anaerobe would possess but the single enzyme, the one which is enabled to work only when oxygen is absent. It is proposed to designate the respiratory enzyme of the aerobe as aerase, and that of the anaerobe as anaerese. This differentiation in physiologic activity would therefore find its basis in the nature of the enzymes present. The toxic action of oxygen on anaerobes would imply the oxidation of its anaerese.

SUMMARY

By manometric means and by analysis it was shown that the raw potato cylinder carried on an active aerobic respiration which, with the disappearance of oxygen, was succeeded by an equally intense anaerobic respiration.

The rate of respiration varied with the temperature. The optimum was at or below 31 C. It was somewhat less active at 34-39 C.

The respiratory mechanism was destroyed by heating the potato cylinders in an autoclave at 120 C. for 15 minutes.

The aerobic respiration resulted in the complete removal of oxygen in the tube, whether filled with air or with pure oxygen. The amount of CO₂ returned was slightly less than that of the oxygen consumed.

In Novy jars, with air, the yield of CO₂, unreduced, was 8-11 c.c. per gm. of potato. The amount of oxygen consumed was slightly more.

The respiratory quotient for the raw potato, in the presence of an excess of oxygen, was on an average 1.006.

The anaerobic respiration which followed the aerobic was indicated by the change in pressure which passed from negative to positive. It was also shown in the high CO₂ content and by the respiratory quotient exceeding 1.

The raw potato when placed in pure nitrogen showed marked anaerobic respiration. A positive pressure developed at once and rapidly increased. The CO₂ content in a tube rose to 24% in 3½-7 days.

No hydrogen or other combustible gas was produced during anaerobic respiration of potato. The test for alcohol was not decisive.

The respiration of the raw potato, when immersed in water or in broth, was characterized by a slight negative pressure which soon changed to positive. This indication of anaerobic respiration was confirmed by analysis. It proved the absence of oxygen in the liquid. The respiratory quotients were high, ranging from 1.5 to 7.6 and even higher.

The immersed potato removed all of the dissolved oxygen and thus created an anaerobic condition in the broth which enabled obligative anaerobes to grow in the medium, apparently in the presence of air.

The distant potato present in the one arm of the *h*-tube enabled the anaerobes to grow in broth in the other arm. This was shown to be due to the complete removal of oxygen.

Anaerobes can be grown in poured plates and on the surface of agar plates by placing nonsterile potato in a Novy jar. The oxygen was completely removed by the combined respiration of potato and aerobes.

The distant culture of *B. subtilis*, in one arm of the *h*-tube, enabled the anaerobes to grow in broth in the other arm. Oxygen was completely removed as in similar tests with distant potato.

By placing a sufficient number of agar plates inoculated with *B. subtilis* in a Novy jar, anaerobes could be grown on the surface of agar plates or as colonies in poured plates.

Anaerobes could be grown in "mixed culture" with the pneumococcus, which is considered to be a peroxide former and to have little or no catalase.

The inability of anaerobes to grow in the air is not due to the hypothetical production of peroxide and to the absence of catalase.

The view is put forth that the fundamental difference between obligative aerobes and anaerobes lies in the nature of the respiratory enzymes, which are designated as aerase and anaerase, respectively. The potato and the facultative anaerobe possess both types; that present in obligative anaerobes can function only in the absence of oxygen, while that of the aerobe can work only in the presence of oxygen.

CORRECTION

In the first article of this series, which was published in the February issue, the formula for reduction of the gas volume to 0 degree and 760 mm., which appeared on page 157, should read:

$$V_0 = \frac{V}{1 + 0.003665 t^\circ} \cdot \frac{B-b-T}{760} = 1315.87 \text{ c.c. at 0 degrees, 760 mm.}$$

PATHOGENICITY OF CLOSTRIDIUM BOTULINUM *

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The pathogenicity of *Clostridium botulinum* has been the subject of much laboratory work with variable results. Botulism in man is unquestionably a true intoxication caused by the soluble toxin elaborated by *Cl. botulinum* under saprophytic conditions. There is a possibility, however, that the organism itself may multiply and produce this toxin within the body, since it is recognized that the temperatures employed in preparing contaminated material for consumption might be insufficient to kill the highly resistant spores of the organism, although rendering the product safe as far as the presence of any preformed toxin is concerned. Recent work on other toxin-producing organisms, such as *Cl. tetani*, has stimulated the effort to determine whether such a condition as we find occurring in latent or idiopathic tetanus occurs also in botulism, owing to the presence of spores.

The earlier workers on botulism established the fact that the disease is an intoxication, and denied that the organism itself is directly involved. This opinion was based on the observation that the organism is incapable of multiplication in vitro at temperatures approaching that of the body of warm-blooded animals. Other workers, while reporting growths, though impaired, at such temperatures, denied the ability of the organism to produce a potent toxin under such conditions. The explanation for these peculiar observations relating to the thermal properties of the organism is not clear. More recent work with *Cl. botulinum* shows plainly that the strains studied in this country all grow well and produce as potent a toxin in vitro at 37 C. as when grown at 18 to 25 C., the range formerly cited as furnishing the temperature at which toxin is produced.

LITERATURE

Since Coleman and Meyer¹ have reviewed recently the literature bearing on this phase of the study of botulism, it does not appear necessary to duplicate their summary. Only such articles as have appeared since the publication of their paper will be referred to specifically, as they

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* This study was aided by a grant from the National Cannery Association.

¹ Coleman and Meyer: Jour. Infect. Dis., 1922, 31, p. 622.

are related to certain phases of the work. The general results as gathered from a review of the literature to date are inconstant. All agree, however, that the organism itself, either spores or vegetative cells, is pathogenic only when extremely large numbers are used.

OBJECTS OF THE WORK

The work reported in this paper deals with various phases of the pathogenicity of *Cl. botulinum* and the factors modifying it. We hope to throw some light on the following questions related to pathogenicity:

1. Are the spores of *Cl. botulinum* (detoxified) capable of germination in the body of warm-blooded animals?
2. If germination and multiplication occur, are they accompanied by the production of a potent toxin?
3. Is there any evidence of latency, as regards germination of the spores?
4. How do the spores distribute themselves within the animal body? How frequently may they be demonstrated?
5. Do we find evidence of toxin in the organs of animals dying of botulism following the introduction of detoxified spores?
6. Is there any evidence of an acquired immunity in animals recovering from repeated injections of detoxified spores, as shown either by the presence of demonstrable antitoxin in the body fluids or by increased resistance of the animals to the toxin of *Cl. botulinum*?
7. Do substances ordinarily considered as interfering with the defense mechanism, e. g., as calcium chloride, quinine hydrochloride, etc., in tetanus, exert any influence on the pathogenicity of *Cl. botulinum* spores?
8. What are the macroscopic lesions found in animals dying with the symptoms of botulism following the introduction of detoxified spores?
9. Does the mode of introduction of the detoxified spores play any part in their pathogenicity?
10. What variations exist, if any, in the different species of animals as regards pathogenicity of the spores?

ORGANISMS USED

The organisms employed in the work were single cell strains of *Cl. botulinum*, whose identity had been established by cultural and serologic tests. The strain used in the greater part of the work was one designated in our collection as M7a². This strain came from the single cell isolation of a

culture originally obtained from the olives causing the Greensburg, Pa., outbreak in 1921. It is type A and sporulates readily. It produces regularly a toxin of high potency in artificial mediums and at a temperature of 37 C. In a few instances, other strains, both A and B, were used. These variations will be noted in their proper connection.

TECHNIC

The cultures were grown in casein-digest-veal-infusion broth at 37 C., for from 3 to 4 weeks, after which time practically all existed in the pure spore stage. Tests were made regularly by the intraperitoneal injection of mice with filtrates to show that such cultures were highly toxic. In some cases, the potency of the toxin was determined also by feeding guinea-pigs.

The spores were removed by centrifuging and washing from 3 to 5 times with sterile salt solution. They were suspended in sterile salt solution in such a way as to make the proper density for counting and injecting. They were then heated to 70 or 80 C. for 20 minutes in order to complete detoxification. This detoxification was carried out immediately before injection of the spores, since Coleman and Meyer have shown that detoxified suspensions tend to become toxic again on standing. To control the efficiency of detoxification, duplicate and triplicate injections of the final supernatant fluid in which the spores were suspended were made into mice. Relatively large injections were made, and proper antitoxin controls were always used. In no instance were results considered if the injected mice showed any indication of intoxication. Such instances were rare.

The number of spores introduced was determined by direct count, using a hemacytometer. While such counts are not accurate, they approach near enough to actual numbers to satisfy all the requirements, since even differences of millions probably do not alter the results.

The technic for securing cultures from the organs of animals that died following injections was planned so as to avoid any extraneous contamination if possible. When working in a laboratory used for other work on botulism, the danger of contamination with spores of *Cl. botulinum* must be recognized. Contamination from the body of the animal may occur also. That the percentage of error from such sources must be insignificant is evident from the absence of *Cl. botulinum* in innumerable transfers and exposures of medium, using a similar technic. The section of the room in which necropsies were to be held was wet down with a 4% solution of lysol. Animals coming to necropsy were washed thoroughly in a similar solution of lysol and allowed to remain some time before being opened. They were tied to necropsy pans previously sterilized and partly filled with a solution of lysol. These necropsy pans were then placed in much larger pans similarly disinfected. All instruments were sterilized by prolonged boiling. Organs from which cultures were to be taken were carefully exposed and their surfaces sterilized by thoroughly searing with a spatula heated in a direct flame. Small sections of the desired organ were then removed with scissors and forceps similarly sterilized in the direct flame. Transfers of these tissues were always made to beef heart medium, covered with sterile petrolatum. Incubation was carried on at 37 C. The period of incubation varied but was never less than 10 days. In a number of instances, repeated tests of the cultures showing growth were made, as will be noted specifically later. All tubes showing evidence of growth and gas production were tested for the presence of *Cl. botulinum* by noting the presence of botulinus toxin as shown by inoculation into mice, properly controlled with antitoxin.

EXPERIMENTAL

The first series of animals receiving injections of *Cl. botulinum* was 20 rabbits injected with spores and vegetative cells primarily for the purpose of stimulating the production of agglutinins and complement-binding bodies. These were large healthy rabbits that had been kept under observation for some time. The weights varied from 1,600 to 2,800 gm. The initial injections consisted of detoxified spores given intravenously. These were followed in a part of the group by injections with vegetative cells. The detoxification was accomplished either by heating at 70 to 80 C. for 20 minutes, or by the addition of an excess of the homologous antitoxin. The efficacy of the mode of detoxification was always determined by intraperitoneal injection of some of the inoculum into mice. The initial dose of spores was 20,000,000. In no instance was there any indication of infection or intoxication following this first injection. Repeated injections of similarly prepared spore suspensions were made until 5 injections had been given, gradually increasing the dosage until finally the animals received 90,000,000. The total number of spores injected during this 16-day period ranged from ninety to two hundred million in the different animals. Five of the 20 animals died at varying intervals of time after the last injection, ranging from 36 hours to 8 days. The symptoms of the animals dying were those of botulism.

Necropsy findings were typical of botulism—i. e., congestion of the lungs, kidneys, liver and suprarenals; constipation, often marked; stasis of the stomach with engorgement of food, such stagnation being accompanied by necrosis of the stomach wall, and rupture; congestion and often actual hemorrhages at the base of the brain. Cultures were taken from the various organs, and growths were obtained in which *Cl. botulinum* was demonstrated (table 1). While a single injection of the spores was without effect, an apparent cumulative action after several injections resulted fatally in 25% of the cases.

Since serologic study showed that the detoxified spores did not possess antigenic properties, the remaining rabbits of the group received intraperitoneal injections of detoxified vegetative cells. The number of injections varied from 4 to 32, spaced at 3 to 5 day intervals over a period of more than 2 months. The total number of such cells introduced ranged from 500 to 950 million. These injections were well tolerated, only 3 animals dying during the entire period. The symptoms in these 3 cases were rather indefinite, although necropsy findings simulated those observed in botulism, and cultures from various organs resulted in the demonstration of *Cl. botulinum* (tables 1 and 2). Five of the animals (7-11) were kept for 4 months without any further injection. At the end of that time, each received 500,000,000 detoxified vegetative cells intraperitoneally. These injections were made at 5 p. m., and by 8 a. m. the following day all the animals were dead. No observations of the symptoms were made. Necropsy showed marked congestion of all the viscera, stasis of the stomach, and hemorrhage at the base of the brain. *Cl. botulinum* was recovered from some of the organs, as is noted in table 1.

While anaphylaxis is suggested by the outcome of this injection, it is not offered as explanation for the peculiar reactions in these 5 animals. That the deaths were not due to an intoxication dependent on free toxin in the inoculum is evident, since quantities of the inoculum in excess of that introduced into the rabbits were harmless for mice when given intraperitoneally.

Seven of the animals receiving these injections of spores and vegetative cells showed no ill effects, and were apparently normal after 9 months.

Summarizing the results, it is found that of 20 rabbits injected with detoxified spores, 3 (1, 2 and 3) died within 8 days after the last injection. Three of the 17 animals surviving the spore injections (4, 5 and 6) died after receiving varying numbers of detoxified vegetative cells. In both these groups, the symptoms and necropsy findings were those of botulism. Five rabbits (7, 8, 9, 10 and 11), reinjected after a 4 months'

TABLE 1
INJECTION OF RABBITS WITH SPORES OF CLOSTRIDIUM BOTULINUM

Animals	No. of Spores Injected in Millions	Injections	Mode	Treatment	Symptoms	Survival	Cultures from						
							Spleen	Liver	Heart Blood	Brain	Kidney	Right Thigh	Cecum
1	240	5	Intra-venous	Anti-toxin	Botulism	Died, 36 hrs.	+	+	+	+	+		
2	70	2	Intra-venous	Anti-toxin	Indefinite	Died, 48 hrs.	+	+	+	+	+		
3	Type B 200	4	Intra-venous	Anti-toxin	Botulism	Died, 8 days	+	+	—	+	—	+	—
4	Type B 220	5	Intra-peritoneal	Anti-toxin	Botulism	Died, 36 hrs.	+	+	+	+	+		
5	240	6	Intra-peritoneal	Anti-toxin	Botulism	Died, 48 hrs.	+	+	—	+	+		
6	920	11	Intra-peritoneal	Heated	Indefinite	Died, 2 mo.	..	+	—	—	+
7	960	8	Intra-peritoneal	Heated	—	Died, 12 hrs.	—	—	+	—	+	+	+
8	940	9	Intra-peritoneal	Heated	—	Died, 12 hrs.	..	—	—	—	—	—	—
9	620	8	Intra-peritoneal	Heated	—	Died, 12 hrs.	—	—	—	—	—	—	+
10	920	9	Intra-peritoneal	Heated	—	Died, 12 hrs.	—	—	+	+	—	—	+
11	900	8	Intra-peritoneal	Heated	—	Died, 12 hrs.	—	—	+	—	—	—	+
12	620	7	Intra-peritoneal	Heated	None	Lived							
13	520	6	Intra-peritoneal	Heated	None	Lived							
14	420	5	Intra-peritoneal	Heated	None	Lived							
15	920	6	Intra-peritoneal	Heated	None	Lived							
16	320	4	Intra-peritoneal	Heated	None	Lived							
17	380	4	Intra-peritoneal	Heated	None	Lived							
18	350	3	Intra-peritoneal	Heated	None	Lived							

rest period, succumbed within 16 hours after this single injection. The cause of death in these animals is unknown. Nine of the animals (12-19, inclusive, and 130) remained normal throughout. *Cl. botulinum* was demonstrated in beef broth cultures of various organs of the dead animals, its presence being determined by the production of the specific botulinus toxin.

The conclusion cannot be drawn from these results that there has been actual multiplication of the organisms introduced, with the production in vivo of botulinus toxin. However, the distribution of the organisms throughout the body of the host, their persistence in a viable form for a relatively long period of time, and the presence of lethal quantities of botulinus toxin, either as a result of actual production in the body or by its liberation from the injected cells, is clearly indicated. The possibility of a fatal outcome in rabbits injected with detoxified spores of *Cl. botulinum* is not to be denied.

RESULTS OF FEEDING DETOXIFIED SPORES OF *CL. BOTULINUM* TO RABBITS

Because of the results obtained by injecting rabbits with detoxified spores of *Cl. botulinum*, it was thought advisable to determine the effect of feeding. Six large healthy rabbits averaging from 1,600 to 2,500 gm. were selected for this experiment. These animals had been kept under observation for some time to be sure of their being normal. The spores were obtained in the usual manner and the numbers determined by the methods previously described.

In order to be sure that the animals actually received the designated number of spores, the syringe containing the organisms was attached to a stomach tube and the inoculum thus introduced directly into the stomach. Following this, the syringe was filled with sterile salt solution, which was introduced into the stomach by the same method, thus making certain that all the spores had been received. The number of spores introduced varied from 75 to 200 million.

Of the 6 rabbits thus treated, 4 died with symptoms typical of botulism, during a period of 8 days, following injection. Necropsies were performed on all, and the findings were characteristic of botulism. Beef heart medium cultures from the different organs were made in the usual way. The cultures were incubated at 37 C., but in no instance in the series were we able to demonstrate the presence of the organism in our cultures after 2 weeks' incubation.

Two of the rabbits, receiving 135 and 160 million spores, respectively, remained normal after more than 2 months' observation. The details relating to this experiment are given in table 2.

RESULTS OF THE INTRODUCTION OF THE SPORES OF *CL. BOTULINUM* INTO GUINEA-PIGS

Since guinea-pigs are more susceptible to the action of the toxin of *Cl. botulinum*, and since this response is much more uniform than in rabbits, it was decided to subject a series of guinea-pigs to the injection and feeding of the spores of *Cl. botulinum*. A series of 18 guinea-pigs, averaging in weight from 250 to 350 gm., were selected for the introduction of the spores. These animals were all normal, having been kept

TABLE 2

INJECTION OF RABBITS WITH SPORES OF CLOSTRIDIUM BOTULINUM. (SPORES HEATED TO 70 C. FOR 15 MIN.)

Animals	No. of Spores Injected in Millions	Mode	Symptoms	Survival	Cultures from						
					Spleen	Liver	Heart Blood	Brain	Kidney	Right Thigh	Cecum
19	200	Intra-venous	Lived							
130	200	Intra-venous	Lived							
131	75	Oral	Died, 48 hrs.	0	0	0	0	0	0	0
132	100	Oral	Died, 5 days	0	0	0	0	0	0	0
133	135	Oral	Lived							
134	160	Oral	Lived							
135	200	Oral	Botulism	Died, 7 days							
136	200	Oral	Botulism	Died, 8 days							

TABLE 3

INJECTION OF GUINEA-PIGS WITH SPORES OF CLOSTRIDIUM BOTULINUM. (SPORES HEATED TO 70 C. FOR 15 MIN.)

Animals	No. of Spores Injected in Millions	Mode	Symptoms	Survival	Cultures from							
					Spleen	Liver	Heart Blood	Brain	Kidney	Lungs	Right Thigh	Cecum
37	60	Intra-venous	Lived								
38	60	Intra-venous	Botulism	Died, 6 days	+	+	0	0	0	-	+	0
39	100	Intra-venous	Lived								
40	100	Intra-venous	Botulism	Died, 7 days	0	0	0	0	+	+	0	
41	30	Intra-peritoneal	Botulism	Died, 7 days	+	...	0	0	0	...	0	0
42	45	Intra-peritoneal	Botulism	Died, 5 days	...	+	0	0	0	...	0	
43	45	Intra-peritoneal	Botulism	Died, 7 days	...	+	0	+	+	...	+	+
44	60	Intra-peritoneal	Botulism	Died, 3 days	...	+	+	0	+	0	+	
45	60	Intra-peritoneal	Botulism	Died, 4 days	+	+	0	0	+	...	0	
46*	60	Intra-peritoneal	Lived								
47	60	Intra-peritoneal	Lived								
48	60	Intra-peritoneal	Lived								
49	60	Intra-muscular	Lived								
50	60	Intra-muscular	Died, 20 days	0	0	0	0	0	0	0	0
51	60	Oral	Died, 48 hrs.	...	+	...	+	+	...	+	
52	60	Oral	Died, 8 days								
53	200	Oral	Died	0	0	0	..	.	0	0	0
54	200	Oral	Paralysis	Died, 5 days								

* Antitoxin was administered to this animal.

under observation for 2 weeks or more. Table 3 shows in detail the modes of introduction, the quantities used, and the results obtained. Twelve of the 18 guinea-pigs receiving the detoxified spores died with symptoms of botulism. Antitoxin controls remained normal. *Cl. botulinum* was isolated from the different organs of the necropsied animals in nearly every case, though not always from all the organs selected. Two of the 4 guinea-pigs receiving intravenous injections, 5 of the 8 receiving intraperitoneal injections, 1 of the 2 receiving intramuscular injections, and all 4 of those receiving the spores orally, died. In 5 of the animals receiving from 60 to 100 million spores, there were no indications of botulinus intoxication during the period of more than 2 months in which they were under observation.

TABLE 4
INJECTION OF RATS WITH SPORES OF *CLOSTRIDIUM BOTULINUM*. (HEATED TO 70 C.
FOR 15 MIN.)

Animals	No. of Spores Injected in Millions	Mode	Survival	Cultures from							
				Spleen	Liver	Heart Blood	Brain	Kidney	Lungs	Right Thigh	Cecum
108	60	Intra-venous	Lived								
109	60	Intra-venous	Lived								
110	60	Intra-venous	Lived								
111*	60	Intra-peritoneal	Died, 11 days	+	0	+	+	+	...	+	+
112	60	Intra-peritoneal	Lived								
113*	60	Subcutaneous	Died, 6 days	0	0	0	0	0	...	0	+
114	60	Subcutaneous	Lived								
115	60	Oral	Lived								

* These rats had symptoms.

RESULTS OF THE INTRODUCTION OF THE SPORES OF *CL. BOTULINUM* INTO WHITE RATS

White rats exhibit marked resistance to the action of the toxin of *Cl. botulinum* as compared with guinea-pigs, mice, and rabbits. They are also much more irregular in their response. In view of this fact, results of the injection of detoxified spores into white rats are of some interest. A series of 8 fully grown rats was given injections of the detoxified spores of the organism. The method of preparation of the material differed in no way from that employed in the preceding experiments and need not be repeated. The details are given in table 4.

Of the 8 rats receiving the spores, only 2 died with symptoms simulating those of botulism. The necropsy findings were typical of botulism. The organism was recovered from the different organs of one of the rats, but from the intestinal tract only of the other. The remaining 6 animals continued normal during a period of more than 3 months.

INFLUENCE OF CALCIUM CHLORIDE AND QUININE ON THE
PATHOGENICITY OF THE SPORES OF CL. BOTULINUM

A survey of the literature reveals many articles dealing with factors that may modify the pathogenicity of those organisms which normally exhibit but slight degree of virulence. Early in the study of tetanus, it was observed that the spores might lie dormant for long periods of time within the body of the host and then become reactivated and germinate, giving rise to the so-called "idiopathic tetanus." Many different factors have been suggested as seemingly instrumental in this reactivation, e. g., variations in temperature, either below or above that normal for the host; the occurrence of traumatic injuries with resulting localized necrosis, thus forming a nidus in which the spores developed; the association of certain other bacteria, sometimes pathogenic, sometimes saprophytic types, e. g., staphylococcus or *B. prodigiosus*; the presence of toxins of bacilli, either the same organism or different organisms, in sublethal doses, as occurs when the toxin of *Cl. welchii* is present with the spores of *Cl. tetani*; certain chemicals, some of which are regularly employed as therapeutic agents, the possibility of whose power to activate spores of toxin-producing organisms thus assuming a place of considerable importance.

Chief among these chemical agents are the easily ionizable salts of calciums, such as the chlorides, nitrates, and acetates, and quinine. These factors taken together have been designated by Bullock and Cramer as "kataphylactic" agents, i. e., agents which are capable of "rupturing" the normal body defenses. The conclusions gained from the work on tetanus are that both the chemical types listed are capable of exerting such action. The mode of action of these factors is variable, but they seem to exert their influence by modifying the resistance of the host rather than by increasing directly the activity of the organism. They probably act by producing localized areas of necrosis, which serve as favorable sites for saprophytic development, and by inhibiting the normal phagocytic activity of the cells of the host, thus permitting the spores to develop.

It has seemed worth while to attempt to find out whether the factors that have been shown to be significant in the case of *Cl. tetani* are also significant in the case of *Cl. botulinum* spores; to determine whether there is a possibility of such factors contributing to the development of injected or ingested spores, with the accompanying production of toxin in such quantities as to be a source of danger. During the progress of our work, an article dealing with the same question by Hall and Davis² appeared. They used calcium chloride as the "kataphylactic" agent. They injected spores of *Cl. botulinum*, detoxified in different ways and introduced by different routes. Then they administered calcium chloride to the injected animals. They came to the conclusion that the administration of calcium chloride into guinea-pigs subcutaneously, intraperitoneally, or intravenously does not modify the pathogenicity of *Cl. botulinum* spores.

TABLE 5
INJECTION OF RABBITS WITH SPORES OF *CLOSTRIDIUM BOTULINUM* PLUS CALCIUM CHLORIDE. (SPORES HEATED TO 70 C. FOR 15 MIN.)

Animals	No. of Spores Injected in Millions	Mode	Amount CaCl ₂ Injected Subcutaneously in Mg.	Survival	Cultures from							
					Spleen	Liver	Heart Blood	Brain	Kidney	Lungs	Right Thigh	Cecum
20	200 (A)	Intra-venous	15	Lived								
21	200	Intra-venous	15	Lived								
22*	200	Intra-venous	15	Died, 8 days	0	+	0	0	0	0	+	
23	200	Oral	15	Lived								
24	200	Oral	15	Lived								
25	50	Lived								
26	15	Lived								

(A) Animal received antitoxin.

* This rabbit had symptoms of botulism.

In our work, the hydrochloride of quinine and calcium chloride were both tested. The animals used were guinea-pigs, rabbits, white rats, and white mice. The reports of work on tetanus have shown that the action of the chemical when used with *Cl. tetani* is independent of the mode of introduction, and that it is effective if given before, at the same time, or shortly after the introduction of the spores. In our work, the spores were introduced by various routes as indicated in the following tables, and the chemical was always given at the same time and with few exceptions either intramuscularly or subcutaneously. In a few instances, the quinine was given orally.

² J. Exper. Med., 1923, 37, p. 585.

CALCIUM CHLORIDE

Chemically pure calcium chloride was used, 1% in sterile distilled water. The toleration dose of the salt alone was determined for each kind of animal used, and the quantity in each case never exceeded one-half of this. The animal. The action of the calcium chloride itself was marked even in the

TABLE 6
INJECTION OF GUINEA-PIGS WITH SPORES OF CLOSTRIDIUM BOTULINUM PLUS CALCIUM CHLORIDE. (SPORES HEATED TO 70 C. FOR 15 MIN.)

Animals	No. of Spores Injected in Millions	Mode	Amount CaCl ₂ Injected in Mg.	Symptoms	Survival	Cultures from							
						Spleen	Liver	Heart Blood	Brain	Kidney	Lungs	Right Thigh	Cecum
55	60	Intra-venous	40	Botu-llism	Died, 6 days	0	+	0	+	0	0	0	0
56	60	Intra-venous	40	Died, 12 days	0	0	+	0	+	0	0	0
57	60	Intra-venous	40	Botu-llism	Died, 3 days	0	+	0	0	+	+	0	+
58	100 (A)	Intra-venous	10	Lived								
59	100 (A)	Intra-venous	10	Lived								
60	100	Intra-venous	10	Lived								
61	100	Intra-venous	10	Paral-ysis	Died, 4 days								
62	100	Intra-venous	10	Botu-llism	Died, 5 days								
63	10	Lived								
64	10	Lived								
65	30	Intra-peritoneal	50	Died, 4 days	..	+	0	+	+	..	+	0
66	50 (A)	Intra-peritoneal	10	Lived								
67	50 (A)	Intra-peritoneal	10	Lived								
68	50	Intra-peritoneal	10	Lived								
69	50	Intra-peritoneal	10	Lived								
70	60	Intra-peritoneal	40	Died, 3 days	0	+	+	+	+	..	+	+
71	60	Intra-peritoneal	40	Died, 4 days	+	0	0	+	+	+	+	0
72	45	Intra-peritoneal	50	Botu-llism	Died, 4 days	..	+	0	0	+	..	0	
73	45	Intra-peritoneal	50	Botu-llism	Died, 4 days	..	+	0	0	+	..	+	
74	60	Intra-muscular	40	Died, 3 days	+	+	0	0	0	+	0	0
75	60	Intra-muscular	40	Botu-llism	Died, 5 days	..	+	..	0	+	0	0	+
76	60	Intra-muscular	40	Died, 6 days	0	0	0	0	0	0
77	50	Lived								
78	60	Oral	40	Died, 3 days	+	0	0	0	0	..	0	0
79	60	Oral	40	Botu-llism	Died, 5 days	0	+	0	0	0	+	0	+
80	60	Oral	40	Died, 2 days	0	+	+	+	0	..	+	0
81	200	Oral	10	Lived								

(A) Animals received antitoxin.

case of small doses, there being considerable local destruction of tissues. The organisms were introduced by the various routes indicated in tables 5-8. injections were made subcutaneously or intramuscularly into the thigh of the Controls were always injected with the largest quantities of the calcium chloride employed.

Rabbits.—Each of 5 large rabbits received 200 million spores, detoxified and heated as in the previous experiments (table 5). The calcium chloride was injected subcutaneously. Only 1 of the 5 rabbits thus treated died. Death occurred in 8 days with symptoms of botulism, and the organism was recovered from the liver and the right thigh, which was the one into which the salt was injected and which exhibited a localized area of necrosis. The material cultivated was from this necrotic tissue.

Guinea-Pigs.—Twenty-seven guinea-pigs averaging from 250 to 350 gm. were used (table 6).

Sixteen of 27 (59.3%) guinea-pigs thus treated died. The symptoms were those of botulism and the organism was recovered from the various organs as indicated in the table. In practically all cases, it was possible also to find the organisms in the areas of necrosis from the injection of the calcium chloride.

TABLE 7
INJECTION OF RATS WITH SPORES OF *CLOSTRIDIUM BOTULINUM* PLUS CALCIUM CHLORIDE,
40 MG. (SPORES HEATED TO 70 C. FOR 15 MIN.)

Animals	No. of Spores Injected in Millions	Mode	Symptoms	Survival	Cultures from							
					Spleen	Liver	Heart Blood	Brain	Kidney	Lungs	Right Thigh	Cecum
116	60	Intra-venous	Died, 13 days	...	+	+	+	0	...	+	+
117	60	Intra-venous	Died, 24 days	...	+	...	0				
118	60	Intra-peritoneal	Botulism	Died, 8 days	...	+	0	0	+	0	+	+
119	60	Intra-peritoneal	Died, 9 days	...	+	0	0	+	+	+	-
120	60	Subcutaneous	Lived								
121	60	Subcutaneous	Lived								
122	Lived								

White Rats.—Of 6 white rats receiving 60 million spores and calcium chloride, 4 died with symptoms of botulism, and the organism was recovered in cultures from the different organs of the body (table 7).

White Mice.—Twenty-eight mice received injections of detoxified spores of *Cl. botulinum* (table 8); 19 (67.8%) died. The symptoms were not definite, but the fact that the controls which received similar treatment plus antitoxin survived is evidence that death was due to botulism. Of 7 mice receiving spores but no calcium chloride, 2 died with indications of botulism. Seven controls receiving the calcium chloride only or calcium chloride plus antitoxin lived. Of 8 mice receiving spores, calcium chloride and antitoxin, none died.

In studying the results and comparing the mortality among animals receiving both calcium chloride and spores with those receiving spores only, the mortality appears somewhat greater in the former. However, the differences are not marked and hardly justify the conclusion that the calcium chloride contributed to any increase of pathogenicity of the

spores of *Cl. botulinum*. In the smaller animals, such as guinea-pigs, white rats, and white mice, the indirect action of the calcium chloride must be considered, i. e., the debilitating action on the general health of the animal due to the necrosis attending the introduction of the calcium salt, and to the toxic substances that must arise from this.

RESULTS OF USING QUININE

The frequent occurrence of tetanus following the injection of a prophylactic or therapeutic agent in malaria induced Semple³ to undertake an experimental study of the influence of quinine on the spores of *Cl. tetani*. He found that the injection of quinine di-hydro-chloride aggravated the pathogenicity of these spores in guinea-pigs and rabbits. The areas of necrosis resulting from such injection were found to have been invaded by the tetanus spores which remained latent, and could be isolated after long periods of time. Francis⁴ showed that detoxified tetanus spores might be reactivated by quinine.

In our study of the literature, no reference to any attempt to perform similar experiments with *Cl. botulinum* has been found. The significance of quinine as a "kataphylactic" agent in the case of *Cl. botulinum* is as great as in the case of *Cl. tetani*, since the use of quinine is the common prophylactic for malaria throughout the world.

Hydrochloride of quinine was dissolved in sterile distilled water in amounts as to yield a 6.5% solution. The toxic dose of the quinine is high, and preliminary tests to establish the lethal limits of the salt itself were carried out. It was found that rabbits weighing from 1,600 to 2,000 gm. would tolerate from 100 to 150 mg. subcutaneously and intramuscularly, while guinea-pigs and white rats would tolerate 50 mg. per 150 gm. of weight without any evidence of intoxication. The quantities used never exceeded half that of the lethal dose of the salt. When larger doses were used, the symptoms described for quinine poisoning developed rapidly, and the animal died in convulsions, muscular spasms, and respiratory inefficiency. The symptoms were different from those of botulism, and there was no danger of confusing the two. As with calcium chloride, the introduction of quinine hydrochloride in the sublethal doses gave rise to a severe local reaction with necrosis of tissues. In a few instances, the quinine was given orally. When administered in this manner, much larger quantities would be tolerated by the animals, probably due to the fact that absorption was slow or incomplete.

³ Scient. Mem. Officers and San. Dept. Govt. India, 1911, 43, N. S.

⁴ U. S. Pub. Health Service, 1914, Hyg. Lab. Bull., 95.

The culture of *Cl. botulinum* used in this series of experiments, as well as in the calcium chloride experiments, was M7a², a highly toxicogenic "A" strain. The spores were obtained and detoxified according to the method previously described. The organisms were introduced by different routes (tables 8 to 11). Controls were always used to check

TABLE 8
INJECTION OF WHITE MICE WITH SPORES OF *CLOSTRIDIUM BOTULINUM* PLUS CALCIUM CHLORIDE OR QUININE HYDROCHLORIDE. (SPORES HEATED TO 70 C. FOR 15 MIN.)

Spores Given	15,000,000 Spores Injected									
	Mg. of Calcium Chloride					Mg. of Quinine Hydrochloride				Anti-toxin
	1.25	2.50	5.00	10.0	20.0	2.50	4.00	5.00	6.50	
Subcutaneous.....Lived..	1
Died....	2	1	...
Intraperitoneal....Lived..	...	1
Died....	1
Intravenous.....Lived..	1	1	1	2	...	2
Died....	...	2	1	2	...	1	1	1
	25,000,000 Spores Injected									
Subcutaneous.....Lived..	...	2	1
Died....	...	4	...	1	1	...	2	1
Intraperitoneal....Lived..	1	1	...	1	1
Died....	1	5	2	...	1	...
Intravenous.....Lived..
Died....

Controls:

- 25 million spores subcutaneously, 2 mice—lived.
- 25 million spores subcutaneously, 1 mouse—died.
- 2.5 mg. CaCl₂ plus antitoxin—lived. (2 mice); given subcutaneously.
- 5.0 mg. quinine hydrochloride (3 mice)—lived, given subcutaneously.
- 25 million spores subcutaneously plus 4 mg. quinine subcutaneously plus antitoxin—2 mice—lived.
- 2.5 mg. CaCl₂ subcutaneously—2 mice—lived.
- 15 million spores intravenously—2 mice—lived.
- 25 million spores intraperitoneally—1 mouse—died.
- 15 million spores intravenously plus 2.5 mg. CaCl₂ subcutaneously plus antitoxin—2 mice—lived.
- 15 million spores intraperitoneally plus 2.5 mg. CaCl₂ subcutaneously plus antitoxin—2 mice—lived.
- 25 million intraperitoneally—1 mouse—lived.
- 15 million intravenously plus 4 mg. quinine subcutaneously plus antitoxin—2 mice—lived.
- 25 million intravenously plus 4 mg. quinine subcutaneously plus antitoxin—2 mice—lived.
- 5 mg. quinine subcutaneously—2 mice—lived.
- 10 mg. CaCl₂, 3 mice subcutaneously—lived.
- 25 million spores intravenously plus 5 mg. quinine subcutaneously plus antitoxin—1 mouse—lived.
- 15 million spores intravenously plus 5 mg. quinine subcutaneously plus antitoxin—1 mouse—lived.
- 15 million spores intravenously plus 10 mg. CaCl₂ subcutaneously plus antitoxin—1 mouse—lived.
- 25 million spores subcutaneously plus 10 mg. CaCl₂ subcutaneously plus antitoxin—1 mouse—lived.

the action of the organism alone and the effect of the largest amount of quinine used.

Rabbits.—Eight normal rabbits, weighing from 1,600 to 2,200 gm., were used in this test (table 9). It will be observed that of the 8 rabbits receiving injections, only 2 (25%) died. In one instance, death was delayed for 3 weeks.

The symptoms were those of botulism and the necropsy findings were corroborative. The other died in 3 days with rather indefinite symptoms. *Cl. botulinum* was recovered in cultures from the necrotic material of the thigh in one case and from the cecum and brain in the other.

Guinea-Pigs.—Of 23 guinea-pigs, weighing from 300 to 450 gm. and receiving quinine hydrochloride and spores of *Cl. botulinum* (table 10), 10 died with the symptoms and findings characteristic of botulism. The period of time between injection and death varied from 2 to 18 days. The organs of the animals yielded positive cultures in all but one case. The organism was present in the necrotic tissue of 4 of the 10 dead animals, although in no instance were the quinine and the spores of *Cl. botulinum* introduced into the same part of the body.

White Rats.—Four of the 5 white rats used died, although death was much delayed, symptoms of botulism not appearing in some animals until after

TABLE 9

INJECTION OF RABBITS WITH SPORES OF CLOSTRIDIUM BOTULINUM PLUS QUININE HYDROCHLORIDE. (SPORES HEATED TO 70 C. FOR 15 MIN.)

Animals	No. of Spores Injected in Millions	Mode	Amount of Quinine Injected in Mg.	Survival	Cultures from							
					Spleen	Liver	Heart Blood	Brain	Kidney	Lungs	Right Thigh	Cecum
27	200 (A)	Intra-venous	15	Lived								
28	200	Intra-venous	15	Lived								
29	200	Intra-venous	15	Lived								
30	72	Oral	65	Lived								
31	72	Oral	65	Lived								
32	200 (A)	Oral	15	Lived								
33*	200	Oral	15	Died, 18 days	...	0	0	..	0	...	+	
34	200	Oral	15	Died, 3 days	0	0	0	+	0	0	..	+
35	15	Lived								
36	65	Lived								

(A) Animals received antitoxin.

* This rabbit had symptoms of botulism.

46 days. More marked local reaction developed in the rats with the dosage of quinine used than in the rabbits and guinea-pigs, and the general condition of the animals became poor.

It is doubtful whether much importance should be attached to these results, as so few rats were used and as other conditions of health were poor. However, the controls receiving similar injections plus the homologous antitoxin survived. *Cl. botulinum* was recovered from the organs of both animals that came to necropsy. Necropsy was not performed on two of the animals (table 11).

White Mice.—Eighteen white mice received injections of quinine hydrochloride and detoxified spores of *Cl. botulinum*. The amount of the quinine injected and also the number of spores given varied (table 8). Of these 18 mice, 11 died, indicating some increase in pathogenicity of the spores when used with quinine hydrochloride, as controls receiving quinine alone, spores alone, and spores plus quinine plus antitoxin remained normal.

With one exception, the results when quinine hydrochloride was used show that this substance, as was the case with calcium chloride, does not modify the pathogenicity of spores of *Cl. botulinum*. Only in the rats

TABLE 10
INJECTION OF GUINEA-PIGS WITH SPORES OF *CLOSTRIDIUM BOTULINUM* PLUS QUININE HYDROCHLORIDE. (SPORES HEATED TO 70 C. FOR 15 MIN.)

Animals	No. of Spores Injected in Millions	Mode	Amount Quinine Injected in Mg.	Symptoms	Survival	Cultures from							
						Spleen	Liver	Heart Blood	Brain	Kidney	Lungs	Right Thigh	Cecum
82	100	Intra-venous	13	Lived								
83	60	Intra-venous	50	Lived								
84	60	Intra-venous	50	Died, 3 days	0	0	+	+	+	..	0	0
85	60	Intra-venous	50	Died, 18 days	+	0	+	+	0	+	+	+
86	100 (A)	Intra-venous	13	Lived								
87	100 (A)	Intra-venous	13	Lived								
88	100	Intra-venous	13	Botu-llism	Died, 9 days	0	0	0	0	0	0	0	0
89	100	Intra-venous	13	Lived								
90	13	Lived								
91	13	Lived								
92	30	Intra-peritoneal	65	Botu-llism	Died, 4 days	..	+	0	0	0	..	0	
93	45	Intra-peritoneal	65	Botu-llism	Died, 5 days	..	+	0	0	+	..	+	
94	45	Intra-peritoneal	65	Botu-llism	Died, 4 days	..	+	+	0	+	0	0	
95	50 (A)	Intra-peritoneal	13	Lived								
96	50 (A)	Intra-peritoneal	13	Lived								
97	50	Intra-peritoneal	13	Lived								
98	50	Intra-peritoneal	13	...	Lived								
99	60	Intra-peritoneal	50	...	Lived								
100	60	Intra-peritoneal	50	Died, 2 days	+	+	+	+	+	..	0	0
101	65	Lived								
102	60	Intra-muscular	50	Died, 16 days	..	+	..	0	0	..	0	
103	60	Intra-muscular	50	Died, 10 days	0	+	+	+	+	
104	60	Intra-muscular	50	Botu-llism	Died, 6 days	0	+	+	0	0	0	+	0
105	60	Oral	50	Lived								
106	60	Oral	50	Lived								
107	200	Oral	13	Lived								

was the mortality any higher than among the animals receiving injections of spores only. This increased mortality was probably due to the toxic action of the quinine itself rather than to any change on the disease-producing powers of the spores.

As regards both calcium chloride and quinine, we find no agreement between their action on *Cl. botulinum* and that which they exert on *Cl. tetani*.

USE OF COLLODION SACS IN THE STUDY OF PATHOGENICITY OF THE SPORES OF *CL. BOTULINUM*

The only reference to such a method of procedure in studying *Cl. botulinum* is by Coleman⁵ which appeared during the progress of our work. He concludes that by the use of collodion sacs he was able to demonstrate that the spores of *Cl. botulinum* germinated in the body of rabbits and guinea-pigs, and that they produced toxin. The toxin did not dialyze through the sacs either in vivo or in vitro.

TABLE 11

INJECTION OF RATS WITH SPORES OF CLOSTRIDIUM BOTULINUM PLUS QUININE HYDRO-CHLORIDE, 50 MG. (SPORES HEATED TO 70 C. FOR 15 MIN.)

Animals	No. of Spores Injected in Millions	Mode	Survival	Cultures from							
				Spleen	Liver	Heart Blood	Brain	Kidney	Lungs	Right Thigh	Cecum
123	60	Intra-peritoneal	Died, 17 days	...	+	+	+	0	..	+	
124	60	Intra-peritoneal	Died, 46 days	+	+	0	..	+	..	0	+
125	60	Subcutaneous	Died, 20 days								
126	60	Subcutaneous	Died, 28 days								
127	60	Oral	Lived								
128	Lived								
129	Lived								

TECHNIC

Various methods of preparing collodion sacs are employed in bacteriologic laboratories, as regards the simple mechanics of their formation, their composition, and the after-treatment, e. g., the methods of modifying permeability and sterilization. There is no doubt that one chief explanation of the discordant results obtained in the use of collodion sacs is the lack of uniformity resulting from differences in their preparation.

Individual sacs prepared by the same method must each be tested for strength and permeability.

In the preparation and standardization of our collodion sacs, two slightly differing methods were used. In the first series, the method described in detail by Gates⁶ was employed, in which the collodion is dissolved in an alcohol-ether solvent containing 25 parts of absolute alcohol and 75 parts of ether. In the later work, the method of Eggerth⁷ was substituted. The

⁵ Jour. Infect. Dis., 1923, 33, p. 384.

⁶ Jour. Exper. Med., 1921, 33, p. 25.

⁷ Jour. Biol. Chem., 1921, 48, p. 203.

principle difference between the two methods is the percentages of alcohol and ether constituting the solvent. In Eggerth's method, 12% collodion (parloidon) is dissolved in a mixture of 70-80 parts by weight of absolute alcohol and 20-30 parts by weight of ether. In both methods, a gelatin capsule sealed to a small glass stem which served as an opening for introducing material into the sacs was repeatedly dipped into the dissolved collodion. After partial drying, the sacs were dipped into 95% alcohol, as described by Gates. The gelatin capsule was removed by dissolving it in hot water. No. 12 veterinary capsules were used to obtain sacs which would hold 5 to 8 c.c. of inoculum.

Sacs were sterilized by suspending in distilled water and heating in an autoclave for from 20 to 30 minutes at 15 pounds' pressure. An unsuccessful attempt was made to sterilize the sacs in 70% alcohol, and the method was discarded.

Sacs were always tested for the presence of leaks before using. The sterile water within the sacs was removed and replaced by a sterile 20% casein-digest broth which was inoculated with *Cl. botulinum*. The inoculated sacs were suspended aseptically in sterile distilled water or sterile broth and incubated at varying temperatures and for different periods of time. The fluid outside of the sac was then tested for the presence of organisms by inoculation into sterile beef heart medium. Later, sacs were tested under reduced pressure equivalent to 15 cm. of mercury, using a suction pump.

Experiments were carried out to determine whether the toxin of *Cl. botulinum* would diffuse through the sacs in vitro. The sterile distilled water was replaced with filtered toxin of high potency, as determined by intraperitoneal injections into white mice. Using aseptic precautions, the sacs were then suspended in tubes containing sterile distilled water, and incubated at varying temperatures—refrigerator, room temperature, and 37 C. The periods of incubation varied from 12 hours to 2 days. While occasional sacs showed the passage of the toxin in detectable quantities, most of the sacs were impermeable to the toxin in vitro, as shown by the injection of large quantities of the dialysate into the white mice. The possibility of small leaks in the sacs cannot be eliminated in those instances in which toxin was demonstrated.

The results of our experiments are in accord with the conclusions reached by Coleman⁵ that botulinus toxin does not dialyze through collodion sacs in vitro. It is true that dialyzability in vivo cannot be determined by experiments in vitro, yet certain evidence which will be presented later indicates that similar results are obtained when the sacs are in the body of the animal.

Rabbits.—The series consisted of 9 rabbits and 5 guinea-pigs. All the animals had been under observation until we were satisfied that they were normal. The rabbits weighed from 1,600 to 2,000 gm.; the guinea-pigs, from 300 to 450 gm. Various strains of *Cl. botulinum* were used, both A and B types being represented.

The organisms had been grown in casein-digest broth for from 3 to 5 weeks, and microscopic examination at the end of that time showed practically 100% spores, only an occasional vegetative cell being found. The broth was centrifuged at high speed, and the sedimented spores were washed several times in sterile salt solution. They were resuspended in sterile salt solution and the number determined by direct counting with a hemacytometer. They were

then heated to 70 to 80 C. for 15 minutes to detoxify them. Mice injected with these heated spore suspensions showed no indications of botulism, thus assuring complete detoxification. The spores were then introduced into the collodion sacs in which from 3 to 5 c.c. of casein-digest broth had been placed. The sacs were handled in such a way as to insure sterility, and introduced into the peritoneal cavities of the animals. No difficulty was encountered in introducing the sacs, and none of the animals showed any ill effects from the operation. No infections occurred, and the incisions healed in a few days. The number of spores introduced into the sacs varied from 40 to 60 million.

Rabbit 58: The sac introduced into this rabbit contained casein-digest broth inoculated with our culture 126.7—a type B organism which is highly toxicogenic. This animal died 50 hours after the introduction of the sac, with typical symptoms of botulism beginning to appear in 36 hours. Necropsy findings showed the sac apparently intact and lying free in the cavity, with no indications of a fibrous reaction. Microscopic examination of the contents of the sac, which was a clear straw color, showed a few spores present, but the great majority of the organisms were actively motile vegetative cells. Inoculation of beef heart medium with sac contents gave positive results for *Cl. botulinum*. Aerobic inoculations were negative. The injection of the contents of the sac into white mice, properly controlled with antitoxin, showed that a potent toxin had been formed by growth of the organisms in the sac. Cultures from tissues of rabbit were negative.

Rabbit 70: The sac introduced contained spores of M7a²—a highly toxicogenic A strain of *Cl. botulinum*. This animal died in 96 hours after the introduction of the sac, with symptoms of botulism beginning in 60 hours. Necropsy findings were typical of botulism. The contents of the sac were highly toxic for white mice. Type A antitoxin protected. This sac was somewhat brittle on removal. Microscopic examination of the sac contents revealed *Cl. botulinum*-like organisms. There were few spores and a great number of vegetative cells. These organisms, when introduced into beef heart medium, grew and produced a potent type A toxin. Cultures from tissues of the rabbit were negative.

Rabbit 53: This animal received spores of 126.7. It died in 96 hours with indefinite symptoms. Necropsy revealed a ruptured sac with some peritonitis. Probably this animal died from other causes than botulism.

Rabbit 71: It received a sac containing spores of M7a² and died in 36 hours. Necropsy revealed a ruptured sac, and a microscopic examination showed a staphylococcus in the fluid remaining in the sac. Death in this case was evidently not caused by *Cl. botulinum*.

Rabbit 67: This is one of the most interesting rabbits in the series. It received a sac containing M7a² spores. The casein-digest broth in the preceding animals was not sugar-free, and the ruptures that were found were probably due to the presence of the gas produced during the growth of the *Cl. botulinum*. In this, and in all succeeding experiments, care was taken to use only broth known to be sugar-free, and no further difficulties were experienced with the rupture of sacs. This animal lived for more than 4 months, being apparently normal all the time. At the end of this time, the sac was removed from the animal apparently intact, though somewhat whitened and brittle. The animal recovered from the operation incident to the removal of the sac and was saved for future testing. The contents of the sac presented the appearance of a clear straw-colored fluid, in which no organisms could be observed microscopically in a hanging drop preparation or in a

stained preparation. Injections of small quantities of this fluid into white mice gave negative results for botulinus toxin. The inoculation of some of the fluid into beef heart medium, however, yielded a growth typical of *Cl. botulinum*, with the production of a potent toxin (type A). Thus while no organisms could be observed, it is evident that viable forms remained in the body of the animal for a period of 4 months. Whether they were the original spores introduced or spores formed as the result of a previous cycle of vegetative cells cannot be definitely determined, although there is some indirect evidence that there had been multiplication. At the time of the removal of the sac, blood was drawn from the ear vein of the rabbit and tested for the presence of antibodies which might have been formed as a result of the organism introduced.

No antitoxin could be detected in the serum when it was tested against the homologous toxin in white mice. Agglutination did occur with the homologous strain of organisms in dilutions as high as 1:200. Since normal agglutinins of such a titer have never been found in normal rabbits, it indicates an active immunization. It is evident that there was some antitoxic protection *in vivo*, since after recovery this rabbit received injections with a lethal dose of type A toxin, which did not produce botulism.

Rabbit 64: This rabbit received a sac containing spores of 126.7 plus 1 c.c. of antitoxin intraperitoneally. The animal remained normal, and the sac was removed 4 months later. It was found firmly bound to the mesenteries by fibrous adhesions. No peritonitis was observed. The sac was removed and examined. It appeared somewhat brittle. Examination of the contents in stained preparations was negative. Cultures in beef heart medium were negative. Injections of the sac contents into mice were negative. Agglutination tests, using serum taken from the rabbit at the time of removal of the sac, were negative.

Rabbit 75: This rabbit received a sac containing spores of M7a². For 10 days, the animal remained normal, after which time symptoms of botulism began to develop, and death occurred on the 12th day. Necropsy showed a normally healed operative wound and no peritonitis. There was marked congestion of the visceral organs; the stomach was gorged with food; there was beginning necrosis of the wall; the gallbladder was distended, and the tissues icteric. Congestion and hemorrhage were noted at the base of the brain.

The sac was found in good condition and was carefully removed. Examination of the contents showed a cloudy fluid which contained clostridial shaped organisms, which were motile and gram-positive. No free spores were noted. Cultivation in beef heart medium yielded positive growth and toxin production. The sac was placed in a tube containing sterile water and allowed to stand 48 hours on ice. The fluid without the sac showed positive results for *Cl. botulinum* on cultivation in beef heart medium. The contents of the sac were tested for toxicity, and it was found that 0.25 c.c. of a 1:5,000 dilution constituted a lethal dose for white mice. Antitoxic controls using homologous antisera remained normal. Cultures taken from the right kidney, right thigh, liver, lungs, and brain were positive, and from the cecum and heart blood, they were negative.

Rabbit 252: This animal received a sac containing spores of M7a² plus 1 c.c. of homologous antitoxin. This animal remained normal, and the sac was removed after a period of 4 months. It was removed in good condition. The fluid contents were clear and yellow. Microscopic examination revealed typical club shaped bacteria, with spores within the cells. Many free spores were also present. Cultures were positive for *Cl. botulinum*, yielding a

TABLE 12
THE INTRODUCTION INTO GUINEA-PIGS OF COLLODION SACS CONTAINING DETOXIFIED SPORES OF CL. BOTULINUM

Animals	Sac Contained Spores of	Sac Remained in Peritoneal Cavity for	Effect on the Animal	Symptoms	Necropsy Findings	Microscopic Examination of Sac Contents	Culture from Sac Contents	Toxicity of Sac Contents	Cultures from the Tissues of the Animals
72	Type B (126.7)	60 hrs.	Died, 60 hrs.	Botulism	Characteristic of botulism; marked distension of stomach with putrefying food; cerebral hemorrhage; sac intact; fibrous adhesions	Vegetative cells resembling Cl. botulinum	Positive for Cl. botulinum	0.25 c.c. is lethal dose for mice; antitoxin protects	Not made
69	Type A (M7a ²)	10 days	Died, 10 days	Remained normal for 8 days; symptoms of botulism developed on 9th day	No peritonitis; characteristic of botulism; sac surrounded by fibrous adhesions	Vegetative cells resembling Cl. botulinum	Positive for Cl. botulinum	Not tested	Cultures from heart blood, liver, right thigh, brain and cecum all negative for Cl. botulinum
60	Type A (M7a ²) 1 c.c. homologous antitoxin given	4 mo.	Remained normal; sac removed after 4 mo.; animal killed	Normal; sac surrounded by fibrous adhesions; somewhat brittle	Nothing; clear straw-colored fluid	Negative	Not tested	Cultures not made; serum tested for agglutinin and antitoxin; both tests negative
73	Type B (126.7) 1 c.c. homologous antitoxin given	4 mo.	Remained normal; sac removed after 4 mo.; animal killed	Normal; sac intact, brittle and embedded in fibrous adhesions	Nothing observed; fluid clear	Negative	Mice injected with sac contents remained normal	Cultures not made from tissues; serum tested for agglutinin and antitoxin both tests being negative
66	Type B (126.7)	Remained normal						

botulinus toxin. Tests for agglutinins and antitoxins in the serum of this animal were made. Agglutinins against the homologous type of organism were demonstrable in dilutions of 1:100, although complete agglutinations did not occur. No antitoxin could be detected in vitro. After recovery from the operation incident to the removal of the sac, the animal was given a lethal dose of type A toxin and died of botulism in the usual time and with typical symptoms, showing that no antitoxin had been produced as a result of harboring the organisms within the sac. The contents of the sac were toxic for white mice.

Rabbit 296: This animal received the same treatment as did Rabbit 252. It remained alive and normal; nothing further was done with it.

Guinea-Pigs.—Sacs containing detoxified spores were placed in the peritoneal cavities of 5 guinea-pigs. Since the method of procedure was identical with that used in the case of the rabbits, the details of this experiment are omitted. Table 12 presents the essential facts pertaining to this series.

The sacs for this series were prepared according to Eggerth's method. All the sacs were tested for leaks and permeability under reduced pressure equivalent to 15 cm. of mercury. They were probably somewhat more impermeable than the ones used earlier. In one experiment, the sacs were coated with a high melting point paraffin, thus rendering them completely impermeable, and the material introduced in such a manner that it would be free from the influence of any body factors except temperature. Both sterile toxin and detoxified spores were used in the different sacs. The spores used resulted from the growth of *Cl. botulinum* in casein-digest broth for 13 days at 37 C. They were washed and detoxified as in the preceding series. The culture used was M7a². Two different methods of sterilizing the sacs were attempted. In order to avoid possible change of permeability and shrinking of the sacs when sterilized in the autoclave at high temperatures, an attempt was made to sterilize one group of sacs by keeping them immersed in 70% alcohol for 48 hours. It was soon discovered that such treatment did not insure sterility. The remaining sacs, filled with and immersed in distilled water, were sterilized in the usual way of autoclaving 30 minutes at 15 pounds' pressure. All sacs were tested for permeability of the toxin and the passage of organisms before their introduction into the body of the animal, and most of them on their removal.

As in the preceding series, it was found that the sacs were impermeable in vitro prior to their introduction into the animal. On the removal of the sacs, their general character was found to have changed. They were white and more brittle, and in many instances, both toxin and organisms were found outside the sacs. This does not necessarily indicate increased permeability, but instead may be evidence of small tears in the sacs.

Exper. 1: Three sterile sacs were rendered further impermeable by immersion into sterile paraffin. In one of these sacs was placed botulinus toxin, 0.0001 c.c. of which was the lethal dose for white mice when introduced intraperitoneally. This sac was introduced into a rabbit. Detoxified spores were placed in the other two sacs. One of these was introduced into a guinea-pig, the other into a rabbit (table 13).

Necropsy of rabbits 190 and 195 after 2 months showed the sacs lying free in the peritoneal cavities with no reaction about them. Guinea-pig 452 died 24 days after the introduction of the sac; the sac was found embedded in fibrous adhesions with marked vascular reactions. It is probable that there was a small leak in this sac, although it was shown by culture that viable

TABLE 13
PARAFFINED SACS CONTAINING STERILE TOXIN OR TOXIN-FREED CELLS

Animal	Sac Contents	Results	Test for Diffusion of		Toxin in Sac	Aerobes in Sac	Leaks	Viability of Organisms in Sac	Presence of Cl. Botulinum in Tissues				
			Toxin	Organism					Spleen	Liver	Kidney	Cecum	Brain
Rabbit 190.....	Toxin	Sac removed after 2 months	0	0	+	0	0	0	
Rabbit 195.....	Cells	Sac removed after 2 months	0	..	+	+	0	
Guinea-pig 452....	Cells	Died in 24 days	+	0	+	+	..	+	0	0	0	Sterile	

TABLE 14
PERMEABLE SACS CONTAINING TOXIN OR TOXIN-FREED CELLS

Animal	Sac Contents	Results	Test for Diffusion of		Toxin in Sac	Aerobes in Sac	Leaks	Viability of Organisms in Sac	Presence of Cl. Botulinum in Tissues				
			Toxin	Organism					Spleen	Liver	Kidney	Cecum	Brain
Guinea-pig 410...	Cells	Died in 4 days	+	+	+	..	Large tear	+
Rabbit 428.....	Cells	Died in 5 days	+	+	+	..	+	+	0	0	Sterile	0	0
Guinea-pig 454*	Toxin	Sac removed after 3 months	+	+	0	..	Sterile	Sterile	Sterile	0	Sterile
Rabbit 881.....	Toxin	Sac removed after 2 months	+	+	Sterile	0	Sterile	0	Sterile

* Injected with sublethal dose of sterile toxin and died in 6 days.

† Injected with sublethal dose of toxin and over 2 lethal doses of toxin 6 days later; died the next day.

organisms still remained in the sac. The contents of all 3 sacs were toxic for white mice.

Under conditions of this experiment, the toxin remained potent within the sac after 2 months. The spores within the other 2 sacs also germinated and produced lethal quantities of toxin in the same time. The absence of reaction about the 2 sacs that were wholly intact is to be noted in contrast with the findings when sacs were not coated with paraffin.

Exper. 2: Four sacs were prepared in the usual way but were not paraffined. Sterile toxin was placed in 2 of them and toxin-free spores of M7a² in sugar-free casein-digest broth in the other. One sac of each group was introduced into the peritoneal cavity of a rabbit; one of each group into a guinea-pig (table 14).

While tears and leaks appeared in the spore sacs, they were not so large as to allow the escape of all the organisms and toxic material. Microscopic examination of the contents of the sac showed the presence of vegetative cells and a few leukocytes. Culture in beef heart medium showed the presence of viable organisms, and injections into mice showed that toxin had been produced. The animals receiving the toxin in the sacs survived, and on their removal the contents of the sacs were still found to be toxic. However, no antitoxic immunity had developed. Marked reaction, fibrous in character, with beginning organization, was observed about the sacs in animals 454 and 88.

Exper. 3: Twelve of the 15 animals received detoxified spores as in the preceding experiment. The sacs were not coated with paraffin. Sterilization was attempted by immersion in 70% alcohol (table 15).

The presence of aerobes within the sacs indicated that sterilization with the alcohol was imperfect, since all cultures had been tested aerobically for sterility prior to their introduction into the sacs. Vegetative cells morphologically resembling *Cl. botulinum* were demonstrable in 9 of the 12 rabbits thus injected. Cultures in beef heart medium showed them to be viable. In 10 of the 12 rabbits, toxin was shown to be present in the sacs after the indicated times.

The permeability tests of these sacs after removal from the body demonstrated the passage through them of both cells and toxin. Whether this was due to leaks which were present while the sacs were in the body or whether it was due to tearing at the time of removal from the body cannot be definitely determined. The finding of the leukocytes within the sacs suggests relatively large openings in them. On the other hand, in the light of our earlier experiences in which the organisms distributed themselves widely and quickly, the large proportion of sterile tissues present would indicate that the leaks must have been small or of recent date.

Three of the animals, guinea-pig 192 and rabbits 496 and 415 were treated similarly but received protective doses of the homologous antitoxin. One of these 3 died from botulism, apparently because of insufficient protection. On removal from the animals after 2 months, the sacs were found to contain toxin no longer. Inflammatory reactions were present around them all, with formation of fibrous tissue and beginning organization.

DISCUSSION

The use of collodion sacs in the study of the pathogenicity of *Cl. botulinum* is accompanied by many difficulties. The lack of uniformity in preparation of the sacs and the variation in permeability makes com-

TABLE 15
PERMEABLE SACS CONTAINING TOXIN-FREED CELLS IN 20% CASEIN DIGEST

Animals	Results	Test for Diffusion of		Toxin in Sac	Aerobes in Sac	Leaks	Viability of Organisms in Sac	Presence of Cl. Botulinum in Tissues				
		Toxin	Organisms					Spleen	Liver	Kidney	Cecum	Brain
Rabbit 459.....	Died in 4 days	+	+	+	+	..	+	Sterile	Sterile	Sterile	0	Sterile
Rabbit 417.....	Died in 3 days	+	+	+	+	..	+	0	+	Sterile	0	Sterile
Rabbit 494.....	Died in 4 days	+	+	+	+	..	+	0	Sterile	Sterile	0	Sterile
Rabbit 188.....	Died in 3 days	+	+	+	+	..	+	Sterile	Sterile	+	0	Sterile
Guinea-pig 94.....	Died in 5 days	+	+	+	+	..	+	0	+	Sterile	0	Sterile
Guinea-pig 129.....	Died in 13 days	+	+	+	+	..	+	+	Sterile	+	0	Sterile
Guinea-pig 448.....	Died in 4 days	+	+	+	+	..	+	Sterile	Sterile	Sterile	0	Sterile
Guinea-pig 145.....	Died in 4 days	+	+	+	+	..	+	+	Sterile	Sterile	0	Sterile

TABLE 16
PERMEABLE SACS CONTAINING TOXIN-FREED CELLS IN 20% CASEIN DIGEST BROTH

Animals	Results	Test for Diffusion of		Toxin in Sac	Aerobes in Sac	Leaks	Viability of Organisms in Sac	Presence of Cl. Botulinum in Tissues				
		Toxin	Organisms					Spleen	Liver	Kidney	Cecum	Brain
Guinea-pig 114.....	Died in 10 days	0	0	+	0	..	+	0	0	0	0	0
Guinea-pig 186.....	Died in 6 days	+	..	Sac torn	..	+	0	+	0	0
Rabbit 199.....	Sac removed after 1 month; died 6 days later	0	0	+	0	0	0	0	0	0
Rabbit 116.....	Sac removed after 6 weeks; injected with toxin 7 days later, and died the next day	0	0	+	0	0	0	0	0	0

parison of results impossible. A fact to be noted is that tests for permeability prior to introduction mean nothing, since it is evident from the study of removed sacs that the collodion undergoes changes within the animal body which make it much more fragile. Tests for permeability following removal give little more reliable information relative to the character of the sacs while within the body, since the mere act of removal may easily result in small breaks in the brittle sacs, thus making possible the passage of organisms and toxin, as was so frequently observed. The fact that there is no widespread distribution of the spores in various tissues, as is the case when cells are introduced directly into the body of the animal; that the contents of the sac still contain the *Cl. botulinum* cells, often in large numbers; and that the contents of the sacs are still highly toxic is evidence that if leaks are present in the body they must be small or of recent origin. If the leaks or tears were large or had been present long, it might be expected that both cells and toxin would have escaped.

On the other hand, the toxin must pass through to a certain extent, either by means of small leaks or by dialysis, since most of the animals not protected by antitoxin died with distinct symptoms of botulism following the introduction of sacs containing detoxified spores.

The escape of the toxin seems in some way dependent on changes in the permeability (or the development of leaks) in the sacs, since many of the animals remained normal for long periods of time and then suddenly manifested symptoms of acute botulism, dying in from 24 to 36 hours. This might indicate a period of latency in the development of the spores. The inflammatory reaction observed about most of the sacs that have remained in the body any length of time, with the exception of those rendered completely impermeable by dipping in paraffin, indicates the passage of some irritant from within the sac. Coleman⁵ made similar observations and suggested that these reactions might be due to the diffusibility of the ammonia salts which are products of *Cl. botulinum* metabolism.

From these observations, it is evident that the exact relationship between the organisms within the sac and the host is not definitely known.

That the detoxified spores of *Cl. botulinum* will germinate and produce toxin in the body of the animal, in the presence of its dialyzable products can, however, no longer be doubted. In repeated instances, microscopic examination of the contents of the sac showed the presence of vegetative cells almost exclusively, and cultures of the same in beef heart medium always yielded typical *Cl. botulinum* growth with toxin

TABLE 17

PERMEABLE SACS CONTAINING TOXIN-FREED CELLS IN 20% CASEIN DIGEST BROTH
Each animal in this series received 4 c.c. of homologous botulinus antitoxin 2 days later.

Animals	Results	Test for Diffusion of Toxin		Toxin in Sac	Aerobes in Sac	Leaks	Viability of Organisms in Sac	Presence of Cl. Botulinum in Tissues				
		Toxin	Organisms					Spleen	Liver	Kidney	Cecum	Brain
Guinea-pig 192.....	Died after 11 days Sac removed after 1 month; toxin injected 7 days later;	+	+	+	0	+	+	0	0	0	+	0
Rabbit 496.....		0	0	Sac torn	0	0	0	0	0	0
Rabbit 415.....	Sac removed after 1 month; toxin injected 7 days later; remained normal	0	0	+	0

production. Likewise, the contents of the sacs were always found to be toxic when injected intraperitoneally into white mice. In a few instances, there was evidence that the cells served as antigens, since agglutinins could be detected in vitro, using the serum of the animal harboring the sacs. Antitoxin could not be detected, excepting in one animal (rabbit 67).

In arriving at any conclusion regarding the pathogenicity of *Cl. botulinum* spores, one must always keep in mind the possibility that the intoxication may have resulted from the liberation of preformed toxin in the spores introduced.⁷ Whether the number of spores injected would contain a lethal dose of such preformed toxin has never been determined. That multiplication occurs, however, is unquestionable, as shown by the presence of vegetative cells in abundance in collodion sacs which had received only limited numbers of spores. Hence the possibility of the liberation of lethal doses of toxin as the result of autolysis of these vegetative cells is in no way an argument against the pathogenicity of *Cl. botulinum* spores.

The viability of the organism when thus introduced into the bodies of animals was found to exceed 4 months.

DISTRIBUTION OF *CL. BOTULINUM* IN THE TISSUES FOLLOWING THE INTRODUCTION OF DETOXIFIED SPORES

Various organs of the animals were cultivated by the introduction of relatively large pieces of tissue into beef heart medium. Table 16 shows the relative frequency of the occurrence of the organism in the different tissues. There does not seem to be any particular tissue in which the organisms tend to localize. Where tissues have been injured, as in the areas of necrosis following the injection of calcium chloride or quinine, the organism was always discovered. No difference in the distribution of spores seemed to be associated with the different modes of introduction.

The number of positive results reported is probably somewhat too low. Especially is this true with organs likely to harbor other organisms, such as normal saprophytes, e. g., the intestinal tract and the lungs. This fact was brought to our notice in connection with our culture work of contents of the large intestines, and may be of considerable importance in all work in which *Cl. botulinum* occurs associated with many other kinds of organisms, as in soils, decaying foods, etc. The usual procedure in testing tissues for the presence of organisms was to make

⁷ Dozier: *J. Infect. Dis.*, 1924, 35, pp. 105 and 134.

inoculations into beef heart medium with a P_H of 7.6 to 7.8 and incubate at 37 C. for from 12 to 18 days, when tests were made for toxin production by injection of mice. In checking results it was found that a large series of cultures had been reincubated after the first test for toxicity. They were allowed to remain in the incubator at 37 C. for from 73 to 89 days, and the filtrates were again injected into mice. It was found that the number of positives was greatly increased. Especially was this true with cecum contents when 20 cultures that were negative on the first test were positive on the second. Similar findings were observed with some of the "lung" cultures. Few differences were observed in cultures from tissues not likely to harbor other organisms, as the heart blood and brain. These findings, in conjunction with some unpublished work on soil examinations, indicate that extreme caution must be exercised when

TABLE 18
DISTRIBUTION OF ORGANISMS IN THE TISSUES

Tissue	Total Examina- tions	No. of Posi- tives	% Posi- tive	Remarks
Spleen.....	20	13	65	In addition, a few cultures were made from the following organs: gallbladder 2, urinary bladder 2, ovary 1, bone marrow 2, small intestine 3, suprarenal 1, spinal cord 2 and thyroid 1
Liver.....	28	23	82	
Heart blood.....	25	11	44	
Brain.....	25	15	60	
Kidneys.....	28	19	67.8	
Lungs.....	28	11	39.3	
Right thigh.....	20	12	60	
Cecum.....	25	17	68	

reporting negative findings of *Cl. botulinum*, especially after short incubation periods. The factors responsible for this latency in toxin production are not known. Carefully done positives are significant. Negative findings mean little.

CHANGES IN ANIMALS DYING OF BOTULISM FOLLOWING THE INTRODUCTION OF DETOXIFIED SPORES

The changes were those commonly described as characteristic of botulism, i. e., congestion of the viscera, lungs, and brain. Certain changes observed in our work have not received much mention in the literature relating to *Cl. botulinum*, and since they were so marked and constant, they seem worth emphasizing: the marked stasis of the contents of the gallbladder, urinary bladder, and stomach. Marked icterus was common, especially in rats. In these cases stagnation of bile was also noted. In rabbits and guinea-pigs, it was common to find the

stomach much distended and gorged with food undergoing decomposition. The stomach wall, especially in the fundus region, was much congested, becoming necrotic, so that the slightest handling resulted in rupture. In some cases, actual rupture had occurred before removal.

CONCLUSIONS

Detoxified spores of *Cl. botulinum*, both A and B types, are capable of multiplication in the body of animals and of producing a potent toxin in amounts sufficient to induce experimental botulism. The number of spores necessary to accomplish this is quite large, and it is doubtful that the introduction of detoxified spores would be of any significance in the problem of human botulism.

The spores of *Cl. botulinum* may remain latent in animals for long periods of time and still be viable. In some instances, viable spores were recovered from the contents of collodion sacs 4 months after their introduction into the body.

Cl. botulinum was isolated from the different organs of the body after death from botulism following introduction of detoxified spores. The distribution was widespread although the highest percentage of positive results was found in the liver, kidneys, spleen, and cecum.

Organs of animals dying from experimental botulism following the introduction of detoxified spores, when macerated in salt solution, never were found to contain botulinus toxin.

Antibody formation agglutinins in animals receiving detoxified spores could be demonstrated. Antitoxins were never demonstrated by testing the serum of such animals *in vitro*, and only in one case by any change occurring in the resistance toward the toxin *in vivo*.

So-called "kataphylactic" agents, such as calcium chloride, quinine hydrochloride, which have been found to modify the pathogenicity of the spores of *Cl. tetani*, did not exert any influence on the pathogenicity of the spores of *Cl. botulinum*, even when given in quantities large enough to produce necrosis.

In addition to the lesions ordinarily described as characteristic of botulism, attention is called to the marked stasis observed in the urinary bladder, the gallbladder, and the stomach, resulting in an icteric condition of the tissues and in necrosis and rupture of the stomach wall.

Collodion sacs were employed successfully in the study of pathogenicity of *Cl. botulinum* and furnished definite evidence of the multiplication and toxin production of the organism in the body of animals.

STAPHYLOCOCCI FROM THE LIVER, GALLBLADDER AND INTESTINE OF NORMAL DOGS

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The bile is usually considered sterile under normal conditions in the vast majority of animals that have been examined, including man. Toida¹ has reviewed the literature, in which one finds many conflicting statements. Rabbits, guinea-pigs and dogs have been used mostly for experimental work. Many authors do not state clearly whether the animals were living or dead at the time the specimens were taken for examination; others do not record the amount of material used for seeding, nor do they mention the culture medium they used. It is known that the intestinal flora ascends into the biliary tract shortly after death. Boardman² studied the bacterial flora of the duodenal contents obtained by the Lyons-Meltzer method. He found that from 74 to 86% of the 56 cases investigated had the same bacteria in the second and third bile aspiration specimens as were present in the mouth and stomach. If Boardman's results are substantiated, much of the previous work on the bacterial flora of the duodenal contents obtained by the Lyons-Meltzer method will have to be revised.

We were interested in studying the bacteria in the cystic bile and mucosa and their relation to the bacteria in the duodenum of healthy dogs. We have also attempted to classify the staphylococci from liver, cystic mucosa and duodenum of healthy dogs in order to determine whether the gallbladder strains were of liver or duodenal origin. We included some strains from the mouth and colon for comparison.

TECHNIC

The abdomen was opened under anesthesia and the liver with the gallbladder exposed, the duct was clamped off with a hemostat, and the liver and gallbladder removed and placed on sterile towels. The gallbladder was swabbed with alcohol; 1 c.c. of bile was removed by puncturing with a pipet and transferred to broth. The gallbladder was emptied by means of an opening in one end with sterile scissors, the bladder slit open under sterile

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¹ Arch. f. klin. Chir., 1913, 103, p. 407.

² Amer. Jour. Med. Sc., 1924, 167, p. 847.

conditions and held spread out firmly with hemostats, while the mucosa was scraped with a sterile scalpel. The scrapings were removed from the scalpel with a sterile swab and transferred to broth. As soon as the bile and mucosa specimens were obtained, the duodenum was exposed and pulled to the surface, a slit made in it about 5 inches from the pyloric sphincter, and a sterile swab inserted into this opening, without touching the sides, pushed toward the pylorus, and a tube of broth inoculated with it. Standard Liebig meat extract broth, with Fairchild's peptone, was used. The reaction was adjusted to P_H 7. For solid medium, 2.5% agar was added. Anaerobic cultures were made in the fluid medium by covering with neutral sterile paraffin oil. Broth was adjusted with brown-thymol-blue, and this indicator was used to test acidity in the cultures. All sugars were made up in concentrated form in distilled water and autoclaved at 15 lbs. for 15 min. The respective sugars were pipetted into the broth tubes to make a 1% concentration and incubated for sterility before they were inoculated. We have found this to give more trustworthy results than heating the sugars in the broth, which usually causes changes in reaction and loss of sugar content. Gelatin liquefaction was determined after 24 hours at 37 C. and 14 days at room temperature. The technic for the reduction of nitrates recommended by the Committee on Bacteriological Technic³ was used. Defibrinated sheep blood (10%) agar plates were used to determine hemolysin production.

All cultures were incubated for 24 hours at 37.5 C. Gram stains were made from smears of the broth cultures at this time. If these were not clear, subcultures were made on the surface of agar plates and Gram stains made of the different colonies that were present. Anaerobic and aerobic cultures were made from each specimen during the first half of the work. No additional information was gained from the anaerobic cultures, and during the last half of the work, only aerobic cultures were made.

Strains used for animal inoculation were plated out on plain agar and single colonies picked; this procedure was repeated three times in order to make sure that we were working with a pure culture. In rabbits, injections were made into the ear vein; in dogs, into the femoral vein.

EXPERIMENTS

Sixty-three dogs were used. In 5 dogs, the bile from the gallbladder contained bacteria; 4 were pure cultures of staphylococcus, 1 was a streptococcus. In all 5 instances, the same coccus was found in the mucosa of the gallbladder and in the duodenal contents. Forty-four of the 63 dogs, or approximately 70%, had bacteria in the scrapings of the mucosa of the gallbladder. In 39 of these 44 dogs the bile was sterile, in the remaining 5 the same organism was found in the bile as was recovered from the mucosa of the gallbladder. In 4 dogs, the mucosa of the gallbladder contained either staphylococci or streptococci, and the duodenal contents were sterile. In 16 dogs, the duodenal contents contained staphylococci, and the cystic bile and mucosa were sterile. We found only gram-positive cocci in the bile and mucosa. In 15 dogs, gram-negative bacilli were found in the duodenal contents. The common duodenal flora contained staphylococci, usually arranged in small clusters, with a marked tendency to diplococcus formation. Our only interest in this flora was to compare it with that of the cystic bile and mucosa.

³ Jour. Bacteriol., 1922, 7, p. 519.

Two strains of staphylococci isolated from bile and 8 strains from the cystic mucosa of dogs were injected intravenously into rabbits. One 24 hour old agar slant culture emulsified in normal salt solution was used in each instance. All 10 strains were nonpathogenic in this dosage.

Four strains from the mucosa were injected in larger doses into rabbits. Two heavily seeded agar slants of each were used for each injection. Two rabbits died after 72 hours; the remaining 2 after 5 days lost considerable weight and were killed. The first 2 animals had abscesses in the lungs and pericarditis. The gallbladder was normal. Staphylococci were recovered from the heart blood and the abscesses (strains M_3 and M_6). The last 2 rabbits had a severe enteritis; no other lesions were found. Staphylococci and a small gram-negative bacillus were isolated from the heart blood.

Strains M_3 and M_6 were passed through other rabbits until 1/20 of an agar slant was the lethal dose within 72 hours. This required 14 successive animal passages for M_3 and 17 passages for M_6 . These strains were then injected (1/20 of agar slant) intravenously into 12 rabbits, 6 rabbits, of 1 kg. weight, receiving the same dosage of each strain. All died between 48 and 72 hours after injection. One rabbit receiving M_3 strain had a severe cholecystitis, with abscesses in lungs and heart; the remaining 11 had only lung and heart lesions; in all instances, the staphylococcus was recovered in pure culture from the heart blood.

Mann⁴ found that the intravenous injections of 8 c.c. of a solution of chlorinated soda (Dakin's solution) per kilogram in dogs produced an acute inflammatory reaction of the gallbladder. There was an infiltration and hemorrhage in the muscularis and between the muscularis and serosa. He gives a photomicrograph of a section of a gallbladder 50 hours after injection of Dakin's solution. There was a small ulceration in the mucosa extending into the muscularis. Mann mentions that such lesions are rarely found.

We found that 10 c.c. chlorinated soda solution per kilogram in dogs gave more constant and uniform results than 8 c.c. or a lesser amount. After we had ascertained the proper dose for our purpose, 10 dogs received intravenous injections with 10 c.c. per kilo. of weight. All dogs were killed 40 hours after injection. The typical hemorrhagic gallbladder described by Mann was found in each dog; in addition, we found ecchymoses under the endocardium, both right and left side, and under the pleura. The gallbladders were removed as in the previous experiments, and cultures were taken from the bile and scrapings of the mucosa. The bile from all 10 dogs was sterile; the mucosal scrapings from 6 gave staphylococci, and 4 were sterile. Sections from various portions of the gallbladders, stained with hematoxylin-eosin and Gram's stain, some cut in serial order, failed to reveal ulcers in the mucosa; the hemorrhage and infiltration of the outer layers were the same as in those described by Mann. We never found a gram-positive coccus in the wall of the gallbladder in our sections.

We were unable to find that *Staph. albus* present in the wall of the gallbladder could be made to produce lesions even after pathologic changes had been caused in the outer coats by intravenous injection of Dakin's solution. We found it difficult to increase the virulence by rabbit passage of these albus strains from the normal cystic mucosa of the dog. Six dogs, each weighing approximately 6 kilos. received injections of *Staph. albus* from the normal cystic mucosa of other dogs. Three agar slants were suspended in 25 c.c. of salt solution and injected into the femoral vein in each dog. Two dogs were

⁴ Ann. Surg., 1921, 73, p. 54.

killed after 24 hours, 2 after 48 hours, and 2 after 96 hours. None of the dogs showed abnormal symptoms. Heart blood immediately after death was negative in each instance. We could not find any gross lesions, and the gallbladder was normal; the bile was sterile, the mucosa of the gallbladder giving growth of *Staph. albus* in 4 of the dogs.

Eighty-five strains of *Staphylococcus albus* were isolated from the liver, gallbladder mucosa, duodenum, mouth, and colon of 27 healthy dogs. Table 1 shows the results obtained in regard to acid production on a group of sugars

TABLE 1
STAPHYLOCOCCUS ALBUS, FERMENTATIVE REACTIONS

Group	Source	No. Strains	Dextrose	Glycerol	Maltose	Lactose	Mannitol
I	Duodenum.....	2					
	Colon.....	2	+	—	+	+	—
	Gallbladder mucosa.....	16					
II	Duodenum.....	6					
	Gallbladder mucosa.....	2	+	+	+	+	+
	Liver.....	12					
III	Duodenum.....	10	+	—	+	+	+
IV	Duodenum.....	2					
	Liver.....	3	+	+	+	+	—
	Colon.....	2					
	Gallbladder mucosa.....	2					
V	Mouth.....	16	+	—	—	—	—
	Colon.....	2					
VI	Gallbladder mucosa.....	2	+	—	+	—	—
	Liver.....	6	+	—	+	—	+

TABLE 2
EFFECTS OF STAPHYLOCOCCI ON GELATIN, NITRATES, AND MILK

Source	No. of Strains	Gelatin Liquefaction		Reduction of Nitrates to Nitrites		Litmus Milk		
		Posi- tive	Nega- tive	Posi- tive	Nega- tive	Acid Only	Acid and Coagu- lation	No Change
Gallbladder mucosa	22	14	8	12	10	2	6	14
Duodenum.....	20	11	9	12	8	10	10	0
Liver.....	21	16	5	18	3	12	3	6
Colon.....	6	3	3	4	2	2	2	2
Mouth.....	16	5	11	10	6	0	0	16

and alcohols. There are roughly 5 groups; the 6th group contains strains that do not fit into the 5 groups. Table 2 shows the results obtained as to gelatin liquefaction, reduction of nitrates to nitrites, and the changes produced in litmus milk. Table 3 gives our results in a form that makes them more comparable to those of other workers. We found that 2 mucosal, 6 duodenal and 6 hepatic strains laked red blood cells; the other strains did not.

Winslow, Rothberg and Parson⁵ studied 100 strains of *Staphylococcus albus*, and found that 47% liquefied gelatin; of the 80 strains of *Staphylococcus aureus*, 67% liquefied gelatin. Hudson⁶ found that 68% of his 82 strains of

⁵ Jour. Bacteriol., 1920, 5, p. 145.

⁶ Jour. Infect. Dis., 1923, 32, p. 297.

Staph. aureus and 55% of 186 strains of *Staph. albus* liquefied gelatin. The other biochemical activities of the two groups are roughly comparable with the gelatin-liquefying power. *Staph. aureus* is more active than *Staph. albus*. Table 3 shows that the albus strains we have isolated when taken as a whole are comparable to those studied by Hudson; they are biochemically more active than those isolated from the air, dust and other sources by Winslow.

The hepatic, duodenal and mucosal (gallbladder) strains resemble closely *Staphylococcus epidermidis* as described by Gordon;⁷ 14 duodenal, 12 mucosal and 15 liver strains, so far as their action on glucose, maltose, lactose, gelatin and nitrates are concerned, resemble *Staph. epidermidis* of Winslow;⁵ 9

TABLE 3
SUMMARY OF CHARACTERISTICS OF STAPHYLOCOCCI

		Source of Strains of <i>Staphylococcus Albus</i>			
		Gallbladder Mucosa, Duodenum, Liver, Colon and Mouth		Gallbladder Mucosa, Duodenum and Liver	
		No. of Strains	Per- centage	No. of Strains	Per- centage
Gelatin liquefaction.....	Positive	49	58	41	65
	Negative	36	42	22	35
Reduction of nitrates to nitrites...	Positive	56	66	42	67
	Negative	29	34	21	33
Litmus milk.....	Positive	47	55	43	68
	Negative	38	45	20	32
Dextrose.....	Positive	85	100	63	100
	Negative	0	0	0	0
Glycerol.....	Positive	56	66	27	43
	Negative	29	34	36	57
Maltose.....	Positive	67	77	63	100
	Negative	18	23	0	0
Lactose.....	Positive	59	68	55	87
	Negative	26	32	8	13
Mannitol.....	Positive	49	58	36	57
	Negative	36	42	27	43

duodenal and 8 mucosal strains fall in his *Staph. candidus* group; 2 mucosal and 6 liver strains are comparable to his *Staph. candicans* type.

The strains from the duodenum, mucosa of the gallbladder and liver of the dogs act more vigorously on various substrata in the medium than those isolated from the mouth and colon. The right half of table 3 shows the biochemical activity of the duodenal, mucosal and hepatic strains as a group separated from the mouth and colon strains. This group approximates the *Staph. aureus* group of Winslow, Hudson and others. Although producing only white pigment, they resemble more the vigorous fermenting and gelatin-liquefying forms of the aureus group.

The passage of living bacteria through the liver into the biliary tract opens up a question that has interested the bacteriologist for several decades. The majority of workers have confirmed the observations reported in 1886 by

⁷ Thirty-Fourth Annual Report of Local Government Board, 1906, p. 384.

Wyssokowitsch⁸ in his classical paper. He concluded that a pathologic lesion is always produced in the organ that has been found to contain living bacteria in its excretion. Dyke⁹ has recently investigated this subject, and comes to the same conclusion. The presence in the liver of the large number of Kupffer cells that are so actively phagocytic has been suggested as an explanation for the presence of living nonpathogenic bacteria in this organ. The bacteria are not toxic enough to cause a marked reaction, and they can get into the biliary tract in a manner similar to that of any nontoxic foreign body.

SUMMARY

Staphylococcus albus was isolated in pure culture from the mucosa of the gallbladder in 70% of the 63 healthy dogs examined. We could not find any relationship between the bacteria in the duodenum and those in the cystic mucosa. *Staph. albus* isolated from the normal cystic mucosa of the dogs was not pathogenic for dogs or rabbits. After increasing the virulency by many passages through rabbits, there was only 1 in 12 rabbits that developed a cholecystitis. Inflammation of the gallbladder in dogs, produced by intravenous injection of Dakin's solution, followed by the injection of these *albus* strains did not increase the incidence of staphylococci in the gallbladder even when cultures from the mucosa showed *Staph. albus*.

Staph. albus from the liver, cystic mucosa and duodenum of healthy dogs are biochemically as active as the usual orange pigment-producing strains.

⁸ Ztschr. f. Hyg. u. Infektionskr., 1886, 1, p. 3.

⁹ Jour. Path. & Bacteriol., 1923, 26, p. 164.

CULTURE MEDIUMS FOR THE GONOCOCCUS *

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This work was undertaken to find, if possible, a simple or selective medium for isolating the gonococcus that could be used as an aid in the diagnosis of chronic gonorrhea.

The great variety of mediums and procedures recommended for cultivating the gonococcus is evidence of the difficulty of growing this organism. It is well known that the gonococcus grows more readily on artificial mediums after a few subcultures have been made. In the present work, the source of cultures has been chronic gonorrhea in women, and I wish to express my thanks to Dr. F. H. Falls for his cooperation. Acute cases, however, have been used to test the value of the mediums and to act as controls for each new lot of medium.

Several mediums described by other investigators have been tried, with modifications, among them blood agar, testicular agar, hormone agar, semisolid agar, and ascitic fluid agar. The cultures were taken with a sterile loop needle and inoculated at once into the mediums.

The gonococcus grows well on blood agar, but cannot be distinguished from colonies of streptococcus, diphtheroids and a few staphylococcus strains. It is therefore of little value in the diagnosis of chronic cases. The testicular agar prepared in a manner similar to the method described by Erickson and Albert¹ has given fair results, but on it it is even more difficult to distinguish the gonococcus from the other organisms, and in addition it is rather difficult to prepare. The hormone agar described by Huntoon² has given good results in acute cases but in chronic cases it has not proven so good. It is more difficult to prepare and very little better than blood agar.

In the early part of this work, the best results were obtained with a semisolid medium and ascitic fluid agar. The cultures were taken with a sterile loop and inoculated at once into the semisolid medium and streaked on ascitic fluid agar plates. The semisolid medium was at first prepared according to the method described by Hitchens.³ In subsequent

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¹ Jour. Infect. Dis., 1922, 30, p. 269.

² Ibid., 1918, 23, p. 169.

³ Ibid., 1921, 29, p. 390.

work, this medium was modified, with somewhat better results. It is made by adding 500 c.c. of water to 1 lb. of ground lean beef and allowing it to stand at a temperature of 37 C. for 48 hours. The juice is then expressed, and to it is added 20 gm. of peptone, 2 gm. of potassium nitrate, and an equal amount of 0.2% agar solution heated and cooled to 60 C. The reaction is then adjusted to plus 0.9 to phenolphthalein. It is then heated in the autoclave at 15 lbs. for 25 min. after which it is filtered, the reaction determined and adjusted, if necessary, and 2 gm. of dextrose added. Best results are obtained when no adjustment is necessary. The reaction may be left between P_H 6.6 and P_H 7, although a reaction of P_H 6.8 appears to be the best. In this medium the gonococcus grows as well, if not better, than many of the contaminating organisms during the first 12 to 18 hours. The growth is limited almost completely to the surface of the medium. Stains were made after 12 to 18 hours' incubation, and if organisms morphologically similar to

TABLE 1
COMPARISON OF RESULTS ON ASCITIC FLUID AGAR, SEMISOLID MEDIUM, AND DIRECT SMEARS

	No. Cultures	Positive Smears	Positive Plates	Positive Semisolid Cultures
Chronic gonorrhea..	436	108	119	87
Acute gonorrhea....	45	43	44	32

the gonococcus were found, pure cultures were secured by streaking on ascitic fluid agar plates. The addition of 1 part of ascitic fluid to 4 parts of this medium gives slightly better results in some instances, but in other cases the streptococci overgrow the gonococcus. The addition of dyes in varying dilutions to this medium has shown no selective action. Among the dyes tested were gentian violet, methyl violet, crystal violet, iodine green, brilliant green, methyl green, basic and acid fuchsin, methylene blue, and eosin. The semisolid medium has proved of most value in cases in which streptococci or diphtheroids are the associated organisms. It is practically impossible to isolate the gonococcus from this medium when the colon bacillus is present. The semisolid medium, while valuable in some cases, has certain disadvantages which tend to limit its practicability. In the first place, it cannot be used by itself, and, furthermore, the odors associated with its preparation are objectionable to some people. The results on this medium are compared to the results on ascitic fluid agar in table 1.

In isolating the gonococcus in chronic gonorrhea, the ascitic fluid agar with certain modifications has given on the whole the best results.

In the early part of this work, in some of the cases the use of the semisolid medium aided materially in securing positive cultures but later the addition of certain carbohydrates and indicators to ascitic fluid agar has given as good results with much less work. Blood serum agar made in a similar manner gives nearly as good results as ascitic fluid agar, when fresh sheep blood serum is used. In my work, there have been fewer contaminations in ascitic fluid agar than in the sheep serum, and I have had no trouble in securing ascitic fluid necessary to carry on the work. The ascitic fluid agar is prepared by adding 500 c.c. of distilled water to 1 lb. of ground lean beef and allowing this to stand over night in the icebox. In the extract from this 10 gm. of peptone are dissolved while cool; to this are added 500 c.c. of 3% agar which has been melted and cooled to 60 C. This is then titrated and adjusted to plus 0.9 to phenolphthalein. Before the agar has cooled sufficiently to harden, it is heated in the autoclave at 15 lbs. for 25 min. It is then filtered through moistened cotton and canton flannel, placed in 100 c.c. amounts in flasks, and sterilized before the agar has hardened from cooling. The reaction is usually near 1.2 plus to phenolphthalein or P_H 6.8. When ready to use, the agar is melted and cooled to 60 C. and then added to 50 c.c. of ascitic fluid, from which the plates are made. Best results are obtained with freshly poured plates, although in a considerable part of the work the plates were made in the evening and used the following morning.

Colon bacilli have been found frequently in cultures from the urethra and cervix, and can usually be distinguished by the size and rate of growth of the colonies. The staphylococci can be distinguished by the more rapid growth, opaqueness and firmer consistency. The diphtheroids are found occasionally, and after 24 hours' incubation are difficult to distinguish from the gonococcus, but by 48 hours the gonococcus colonies are slightly larger, less opaque, and much softer.

The addition of the several dyes mentioned in connection with the semisolid medium, with the exception of iodine green, have not shown any selective action or proved of value in isolating the gonococcus in chronic cases. The addition of iodine green, as reported by Torrey,⁴ apparently stimulates the growth of the gonococcus on this medium. The dilution for best results lies between 1 : 30,000 and 1 : 50,000. In this medium, the diphtheroids are inhibited and appear as small dry colonies. The staphylococcus, especially albus strains, appears to be inhibited slightly and is not tinted green as is the gonococcus. The addition of iodine green is

⁴ Ibid., 1922, 31, p. 125.

therefore of value in some cases, but in my work the streptococci have been found most frequently.

Since the streptococci have been found so frequently, and as some of them are so difficult to distinguish from the gonococcus, it seemed necessary to study at least one strain in some detail. This streptococcus is gram-negative in direct smears, as well as in cultures, and has no doubt led to mistakes in the diagnosis of chronic cases. It occurs often in pairs or short chains of pairs, and the cocci are slightly flattened on the tangential sides, but they are slightly smaller than the gonococcus. The colonies on ascitic fluid agar are usually slightly smaller in 48 hours and more firm in consistency than the gonococcus. Milk is slightly acidified without coagulation or reduction in two weeks. Gelatin is not liquefied, and indol is not produced. Growth on potato, when there is any, is scant. It is inactive on blood agar, but after several weeks' cultivation on blood, a slight greenish zone occurs around the colonies after from 3 to 4 days.

A modified ascitic fluid agar has been prepared to determine the production of acid by the streptococci and other organisms found in urethral and cervical cultures. The agar is prepared as previously described in 100 c c. amounts, and before melting it for use, 0.5 gm. of the carbohydrate is added and 1 c c. of a 0.04% solution of brom cresol purple. After the agar is melted, it is cooled to 60 C. and added to 50 c c. of ascitic fluid, and plates poured. Fourteen strains of the gram-negative streptococcus have produced acid in dextrose, lactose, maltose, sucrose, levulose, galactose, raffinose, mannite, and glycogen, but not in inulin, salicin, and dulcitol.

All strains of gonococcus isolated have fermented dextrose, and all strains tested (87 in number) have not produced acid in levulose, lactose, galactose, saccharose and mannite. A few strains (11 in number) have not formed acid in salicin, raffinose, inulin, glycogen, cellose and fucose. Six strains of the gonococcus produced slight amounts of acid in maltose on the first subcultivation, but in subsequent tests they failed to form any acid.

All other organisms isolated from cervical and urethral cultures which form colonies resembling the gonococcus have produced acid in levulose and maltose. A few strains of micrococci have not fermented galactose and saccharose. Other carbohydrates have been fermented less often by diphtheroids and other contaminating organisms. Levulose has proved to be the carbohydrate of choice, since a few irregularities were observed in the maltose.

Several indicators were used in testing for acid production. Methyl red was changed by a few strains of colon bacilli. Thymol blue and brom thymol blue apparently did not indicate acid production in many cases. Andrade's indicator has given results almost similar to those with brom cresol purple, but in the former, the medium around the acid-producing colonies becomes clouded rather than colored. In the medium containing brom-cresol-purple, there is a definite yellow tint surrounding the acid-producing colonies. This yellow color diffuses out through the medium, and if acid-forming colonies, as those of streptococci, are near the colonies of gonococci, the latter colonies are surrounded by the yellowish medium, making it impossible to determine which colony produced the acid. In such cases, both colonies are picked to new plates of the same medium, and the reaction observed after 24 to

TABLE 2

COMPARISON OF RESULTS IN SMEARS AND ASCITIC FLUID AGAR CONTAINING LEVULOSE AND BROM-CRESOL-PURPLE

	Positive Smears	Positive Cultures	Positive Smears with Negative Cultures	Positive Cultures with Negative Smears
Chronic gonorrhea, 232 cases...	65	86	6	24
Acute gonorrhea, 29 cases	28	28	1	1

48 hours. If the colonies from cultures streaked on the plates are well scattered, the colonies of gonococci can easily be picked, but if the colonies are numerous and close together, it becomes necessary to pick all suspicious colonies to new plates. This does not require a great deal of time or medium when at least 10 suspicious colonies can be picked to one plate. The results obtained with this medium are compared with the smears in table 2.

CONCLUSIONS

The addition of levulose or maltose and the indicator brom-cresol-purple to ascitic fluid agar gives a medium on which the gonococcus grows readily and can be distinguished easily from other associated organisms.

By the use of the mediums described, discharges in cases of chronic gonorrhea that were considered free from gonococci on the examination of smears, were found to contain gonococci in cultures.

A CHRONIC INFECTION WITH *B. WELCHII*

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It is well established that *B. welchii* plays an essential rôle in the production of gas gangrene, and that contributory factors, trauma and mixed infection, are frequently present. Experimentally, in the absence of mixed infection, a pure culture of *B. welchii* injected subcutaneously into a susceptible animal (e. g., a guinea-pig or pigeon) will produce definite hemorrhagic edema with gas formation, if a culture of sufficient growth and virulence is used. But if a less susceptible animal (e. g., rabbit) be inoculated subcutaneously with a less numerous and less virulent culture, the local reaction is insignificant, gas formation is too slight to be recognized, and the animal shows no marked symptoms. Nevertheless, it is inaccurate to conclude that the animal has overcome the infection. A superficial examination of such an inoculated rabbit during the first week might indeed lead to such a conclusion. But a prolonged and careful examination over a period of 3 months or more reveals that the animal, while showing no initial acute symptoms, is nevertheless suffering from a chronic infection. This statement is based partly on symptoms and chiefly on the ability to recover *B. welchii*, usually in pure culture, from various tissues immediately after death. Of 56 rabbits inoculated at various sites with *B. welchii*, only 1 failed to show symptoms and yielded negative cultures of its organs. The rest have either died or still show symptoms, and in all cases in which cultures have been made at necropsy, the organism has been recovered.

Various strains were used, mostly from human stools, some from infected wounds, as well as one of Bull's original virulent strains. The strains were colony-picked from anaerobic blood-agar plates and identified by suitable methods. During the course of the investigation, care was exercised to keep the strains uncontaminated, and for this purpose frequent platings and smear examinations were made. It was found that the virulence of strains isolated from stools soon declined and required occasional passages through pigeons to improve them.

In the first experiments, the culture was injected into the spleen as a routine method of inoculation.

TECHNIC

With the rabbit under ether, the abdomen was opened, the spleen drawn up into the wound and packed off with sponges of warm iodine water. The needle of the syringe was inserted at the tip of the organ and passed along the long axis, and the culture injected. Usually the fluid portion alone (0.5 to 1 c.c.) of a 24-hour aerobic culture on Robertson's meat medium was used. Before withdrawing the needle, a loop of plain catgut was placed about the tip of the spleen and tied, as the needle was withdrawn, to prevent leakage. Before replacing the organ, the site of the needle puncture was seared with a hot iron to sterilize the surface. Finally, the abdomen was closed with chromic catgut.

Rabbits inoculated in this manner may die within 1 or 2 days, or, on the other hand, live for longer periods varying all the way from a week to 3 or 4 months. Two typical examples are given:

Typical Protocol 1: Short Survival.—A healthy 4 lb. rabbit; hemo., 64%; red corpuscles, 7,760,000; leukocytes, 7,200; smear, normal.

Jan. 29, 3:50 p. m.: Injected into the spleen 0.25 c.c. of culture of a strain isolated from human stool.

Jan. 30, 4:00 p. m.: Animal ill; rectal temp., 101.2 F., eating very little. Hemo., 50%; red corpuscles, 6,464,000; leukocytes, 11,000. Some crenations and variations in the size of the erythrocytes.

Jan. 31; Animal had diarrhea and was very ill, and at 9:40 p. m. it stiffened out into opisthotonos and died.

Necropsy (15 minutes later): Rigor present; buttocks and tail soiled with feces; lungs normal; heart normal; liver deeply congested; spleen deeply congested; suprarenals slightly pink; marrow red, having lost the purplish normal color; cultures showed liver, bone marrow and brain sterile; the spleen gave a heavy, pure growth of *B. welchii*; the skeletal muscle (leg) also gave pure *B. welchii*.

Typical Protocol 2: Long Survival with Death.—Before inoculation, wt. 4 lbs. 2 oz.; hemo., 77%; red corpuscles, 5,288,000; leukocytes, 15,400; smear, normal.

Oct. 26: Injected into the spleen 0.5 c.c. of culture of a strain isolated from human stool.

Nov. 5: Hemo., 53%; red corpuscles, 4,240,000; leukocytes, 17,600; some anisocytosis; wt., 3 lbs. 10 oz.; seems well.

Dec. 11: Hemo., 53%; red corpuscles, 5,376,000; leukocytes, 12,400; some anisocytosis; wt., 3 lbs. 6 oz.

Dec. 26: Hemo., 52%; red corpuscles, 5,352,000; leukocytes, 12,800; some anisocytosis; wt., 3 lbs. 1 oz.

For past week animal had been abnormally excitable and had had some diarrhea.

Jan. 5: Convulsion; the rabbit stiffened into rigid, tetanic opisthotonos for 45 seconds, and then immediately recovered. Diarrhea profuse all day, both before and following convulsion.

Jan. 6: Diarrhea continuous; hemo., 72%; red corpuscles, 8,192,000; leukocytes, 17,000 (increased counts due to dehydration).

Jan. 7: At 5 a. m. animal was seized by convulsion and died.

Necropsy (30 minutes later): Rigor extreme; lungs normal; heart muscle slightly pale; liver, while containing no cysts or gross anatomic abnormalities, presented a diffuse graying of the surface.

Subcutaneous inoculation was found to produce the same effects, although the blood changes were usually more delayed.

Typical Protocol 3: Subcutaneous Inoculation Followed by Long Survival and Death.—Wt., 3 lbs. 15 oz.; hemo., 64%; red corpuscles, 6,824,000; leukocytes, 6,000; smear, normal.

Nov. 5: Inoculated subcutaneously with 1 c.c. of a culture of *B. welchii* isolated from human stool.

Dec. 26: Hemo., 32%; red corpuscles, 3,584,000; leukocytes, 8,000. Smear shows slight variation in the size of the erythrocytes.

Jan. 3, 4, 5: Convulsions.

Jan. 10: Hemo., 45%; red corpuscles, 2,504,000; leukocytes, 9,600. Diarrhea. Weight, 3 lbs. 2½ oz.

Jan. 26: Diarrhea recurred. Hemo., 38%; red corpuscles, 5,024,000; leukocytes, 9,000. Convulsion, in which animal died.

Necropsy: Lungs and heart normal; liver showed no gross change; spleen showed staining; suprarenals pale; no red staining of skeletal muscles; kidneys apparently normal; marrow showed reddening; microscopically, hyperplasia of blood-forming tissue.

The spinal meninges showed some fibrous thickening but no inflammatory reaction microscopically; occasional gram-positive bacilli like *B. welchii*; cord apparently normal; no organisms.

Cultures of spleen, marrow, and skeletal muscle gave pure *B. welchii*; liver, sterile.

Protocol 4.—Inoculated Oct. 26, 1923, into the spleen; died Jan. 6, 1924, with mild anemia, anisocytosis, convulsions, and great loss of weight. After death there was marked red staining of skeletal muscles, distention of stomach, no free HCl; liver had general grayish appearance on surface, section apparently normal; spleen, small, dull brown areas; heart, rather paler than normal; cultures of *B. welchii* from spleen, bone marrow and liver. Spinal cord in section showed meningeal thickening, but no inflammatory reaction; numerous gram-positive bacilli in the meninges at all levels; cord itself apparently normal.

While other sites of inoculation (e.g., bone marrow, muscle) may be used to produce chronic infections, intravenous inoculation does not appear advantageous. Inoculation of *B. welchii* culture into the spinal canal produced interesting results, as shown in protocol 5.

Protocol 5: Intraspinal Inoculation Followed by Paralysis and Death.—Needle inserted well into the spinal canal and 0.5 c.c. of a strain of *B. welchii* isolated from human stools injected; 30 minutes after operation, animal, having recovered from ether, got up and walked normally and continued to walk normally for 27 days, thus indicating that no gross damage had been done at the time of the injection. On the 28th day, animal was found with paralysis and exaggerated knee reflexes—a clinical picture of transverse myelitis. During the next 6 days, these symptoms remained, while gradually there appeared paralysis of the front legs, then of the neck, with death on the 34th day after inoculation. There were marked loss of weight and characteristic blood changes.

After death, an open abscess was found over the 8th dorsal spine with caries of bone; no macroscopic lesion of cord; dura intact and shiny. Sections of cord well above, immediately below 8th dorsal spine, and 2.5 cm. below, stained with hematoxylin and eosin, showed large swollen axonal processes in anterior and lateral white columns, with appearance of degeneration; gray matter normal at all levels. Gram stain revealed bacilli in meninges, none in cord. Marchi preparations of cord above 8th dorsal spine showed severe diffuse degeneration, undegenerate axons among and between degenerate ones. Axonal processes larger and degeneration more marked at periphery; gray matter normal. Imme-

diately below the level of 8th dorsal spine, same general appearance, but with a normal area including the whole of the ground bundles of the anterior and lateral columns of one side; small degenerating axons deeper than the normal area. Sections of peripheral nerves show a moderate percentage of degenerating axons. Lower down was a ring of degeneration of uniform thickness around the periphery of the cord involving the area of large axonal processes; deeper white matter and the whole of gray normal.

For routine purposes, the subcutaneous and intrasplenic sites have been used. Of 56 rabbits inoculated, 30 died within 3 weeks after inoculation, while 26 have survived for periods varying from 3 weeks to 4½ months. Only one rabbit has failed to show symptoms, and the organs gave negative cultures. All the rest have shown symptoms (blood changes, or convulsions or loss of weight or all combined), and, in all those on which cultures have been made (25), *B. welchii* has been recovered.

GENERAL SUMMARY OF THE PICTURE AND LESIONS OF THE INFECTION

A rabbit chronically infected with *B. welchii* shows a fall in hemoglobin such that the normal level is not regained, except terminally when dehydrating diarrhea may occur. The erythrocytes show a variable behavior in their numbers and a constant phenomenon of anisocytosis. The alterations in the leukocyte counts do not follow any regular plan. Most chronically infected rabbits show one or more convulsions of a tetanic character, usually, but not always near death. Loss of weight is, on the whole, irregular and varies from 3% to 25% with an average of 15%. Terminally, severe diarrhea usually appears and lasts until death.

Rigor sets in within 20 or 30 minutes after death, often earlier. Gross examination of the organs shows few changes. An almost constant change is a red color of the marrow, distinct from the slightly purplish normal color. The liver, spleen and abdominal organs are often congested, but apart from this, as a rule, show no striking alterations. Sometimes the liver shows a diffuse graying of the surface with minute areas of brownish red distributed over it. Sometimes mild fatty degeneration of the organ is indicated by its pale and slightly yellowish color. In one case, there was extreme fatty degeneration of the liver, heart and kidneys. Two rabbits showed moderate fatty degeneration of the cardiac muscle alone. The suprarenals are usually pale but may be pink, especially when the animal has died with subacute symptoms. The spleen may be slightly enlarged or seemingly slightly smaller than normal, may have a red as distinct from its normal purplish color, or may,

especially in cases inoculated intrasplenically, be of a dark muddy color. The spleen, if bacteriologically sterile, is normal in color. In 3 cases, the skeletal musculature showed a definite intensification of the red color, a phenomenon that was associated with only one constant factor, viz., marked loss of body weight. Microscopically, in addition to *B. welchii*, meningitis and the occasional fatty degenerations mentioned, the only striking features are those of the bone marrow. This tissue shows greatly increased erythroblastic activity, the medullary fat cells being about half replaced by active hemopoietic structures, the cellular elements of which show as many megaloblastic as normoblastic erythrocytes.

SUMMARY

A chronic infection with *B. welchii* has been produced in rabbits, with mild anemia, loss of weight, convulsions and diarrhea.

BACILLUS GONIDIAFORMANS (N.SP.)

AN HITHERTO UNDESCRIBED ORGANISM

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The organism here described was isolated from an actinomycosis-like tonsillar granule similar to those studied by Davis.¹ Two strains were isolated, but one died before it could be completely studied.

The following methods of fixation and staining were employed:

Some specimens were fixed wet, according to the method of Dobell.² A drop of broth culture and a drop of either 2% osmic acid or formalin were placed side by side on the slide, then thoroughly mixed and spread, and allowed to dry in the air, after which they were fixed further for 10 minutes in absolute alcohol. A saturated solution of bichloride of mercury was used in the same manner. Occasionally also Regaud's fluid, as recommended by Cowdry,³ which is a mixture of 4 parts of a 3% aqueous solution of potassium bichromate and one part of commercial formalin, was used. More frequently, smears were dried in the air and fixed in methyl alcohol for 1-2 minutes. After any of the above methods of fixation, Giemsa's stain in a dilution of 1:10 or Wright's stain gave excellent results. Pappenheim's methyl green pyronin and iron hematoxylin were also tried but seemed to possess no advantages over the other methods.

Satisfactory results were obtained by staining the organisms vitally with brilliant cresyl blue. A thin film of the dye was made by placing a small amount of an alcoholic solution on a slide and spreading it and allowing it to dry. A drop of a broth culture of the organism was then placed on the film and a cover slip applied. Weak aqueous solutions of brilliant cresyl blue, neutral red and Janus green were also employed, but did not bring out details in structure.

The body of the organism does not retain Gram's stain, but the granules inside do. The organisms are not acid-fast. Stained by Giemsa's or Wright's method, the cytoplasm of all forms of the organism stains blue, while the granules and what we have designated chromatin

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¹ Jour. Infect. Dis., 1914, 14, p. 144.

² Quart. Jour. Micros. Sc., 1911, 56, p. 395.

³ Jour. Exper. Med., 1923, 37, p. 431.

masses and network stain a deep red. We were unable to demonstrate fat in these organisms by staining with sudan III, or glycogen by treating them with equal parts of Lugol's solution and glycerol.

That the forms of the organism described are fairly constant, is shown by the fact that they are easily demonstrated by all of the methods employed.

MORPHOLOGY

This organism is extremely pleomorphic. The most frequently observed and stable form is that of a short bacillus with rounded ends (fig. 1) which varies from 1-3 mikrons in length and is about 0.5 mikrons in width. In films, these rods stain light blue with usually a deeply stained red chromatin granule at either end with Wright or Giemsa stain. Associated with these bacillary forms are small round or oval bodies, the smallest about 0.5 mikrons in diameter, which appear to be made up almost entirely of a single chromatin granule. These coccoid bodies we regard as gonidia. As they increase in size, the chromatin divides, and we have bodies about 1 mikron in diameter, with first 2 chromatin granules, then still larger ones with 4 granules, and finally large coccoid bodies 10-12 mikrons in diameter, with varying numbers of regular or irregular chromatin granules or masses (figs. 2 and 3). These large globular forms, which we believe are gonidangia, apparently give rise to gonidia as ruptured organisms are often seen discharging numerous small bodies. These ball-like bodies sometimes appear to have a capsule, but are not resistant to heat.

Instead of the gonidia inside the bacilli dividing, they may enlarge with the rest of the cell, until a cell is formed with a diameter of 5-15 mikrons, containing one or two large, round, deeply staining chromatin masses, those with one resembling lymphocytes (figs. 3 and 4). The chromatin may become arranged irregularly in coarse strands which form a reticular mass and may form into balls which are seen in the process of unwinding (fig. 5) into long or short wavy filaments, some forming perfect corkscrews. The greater part of these filaments appear to be made up of chromatin, staining pink with Giemsa. Chromatin in the form of spirals may also be seen both inside and outside of the filaments.

In some of the early cultures, there are many similar bodies, only smaller, deeply blue stained, irregularly shaped, varying in diameter from 1-4 mikrons and containing variously shaped masses of chromatin. They usually occur in closely arranged groups, the individuals composing each group being approximately the same size. These forms, except for

the absence of pigment, are suggestive of certain forms of the malarial organism. Associated with these irregular forms are numerous bacilli and filaments which have a beaded appearance on account of the regular arrangement of the chromatin into round granules. Groups of small, round, chromatin granules, with a barely visible amount of cytoplasm surrounding each, suggest that they are gonidia which have been discharged from bacilli and filaments, and are the probable source of the larger irregular bodies.

The short bacillary forms may propagate as such for several generations and then give rise to long bacilli and straight and wavy filaments of greatly varying length and width. The slender bacilli and filaments



Fig. 1.—Forty-eight hour growth in ascites phosphate broth, showing short bacillary and filamentous forms. Giemsa's stain; $\times 1,200$.

frequently present the appearance of chains of streptococci on account of the regular arrangement of the chromatin granules. In the thicker filaments, the chromatin granules may be of uniform size and evenly distributed, or the distribution may be patchy with portions entirely devoid of chromatin, and the chromatin masses may be of various sizes and shapes. The large thick filaments often develop pyriform enlargements which may reach a diameter of 8 mikrons, and branching is often observed. The budding of round bodies from the sides of filaments is conspicuous. These bodies are also seen developing within and at the ends of filaments. Budding from the chromatin masses may be observed in almost every form of the organism.

We have been unable to convince ourselves of the presence of the symplastic stage, described by Löhnis,⁴ in our cultures, although in some cases the appearances were suggestive.

The addition to mediums of sodium hydroxide and acetic acid in varying concentrations produced no morphologic changes in any of the forms.

When observed by dark-field illumination, the small bodies inside the large round cells were seen to possess a distinct Brownian movement. All of the different forms seen in the cultures could be demonstrated in the dark field. It was sometimes difficult to distinguish the internal structure of the large forms, probably on account of the capsule.

CULTURAL PROPERTIES

The organism is an anaerobe, growing aerobically generally for one generation only, in the bottom of tubes of fluid mediums; it multiplies at 36 C. after 3 or 4 days' incubation, occasionally in 24 hours; it grows after 6 days' incubation at room temperature. The different forms of the organism are nonmotile; they resist drying 4 hours and are killed by heating at 54 C. for one half hour.

Cultures were made anaerobic according to Wright's method by saturating the cotton stopper with a 5% solution of sodium hydroxide; a piece of pyrogallic acid the size of a pea having been placed on the plug, the tube was closed with a cork and sealed with paraffin.

The colonies on blood agar and ascites phosphate agar appear thick, whitish, slightly moist, with regular edges, varying from the size of a pin point to 1.5 mm. in diameter. In ascites dextrose agar shake cultures, the colonies appear lenticular in shape, at first colorless, later brownish.

Growth does not always appear when first isolated on solid medium, probably on account of the low resistance to drying. In fluid mediums, the growth is flocculent.

This organism grows slightly in plain and dextrose broth, but not generally in subcultures; for satisfactory multiplication, it requires some body fluid. It grows on Loeffler's blood serum. Ascites dextrose and ascites phosphate broth were found most useful for its cultivation. No growth was observed on potato, gelatin or milk. A little gas is generally produced in cultures containing dextrose. No fermentation was seen in Hiss' serum water medium of 1% dextrose, salicin, mannite and inulin.

⁴ Studies upon the Life Cycles of Bacteria, Part I. Review of the Literature, 1838-1918, 1921.

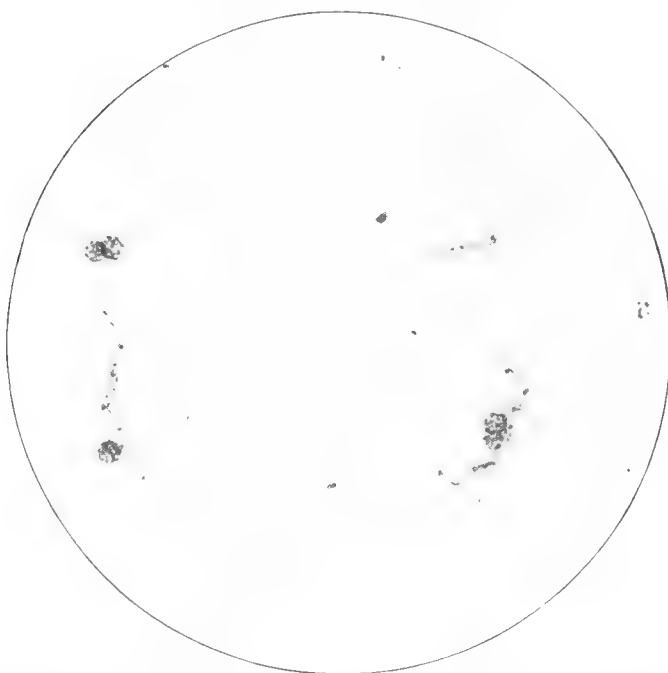


Fig. 2.—Four days' growth in ascites phosphate broth. The specimen shows filaments with gonidangia attached and free. Giemsa's stain; $\times 700$.

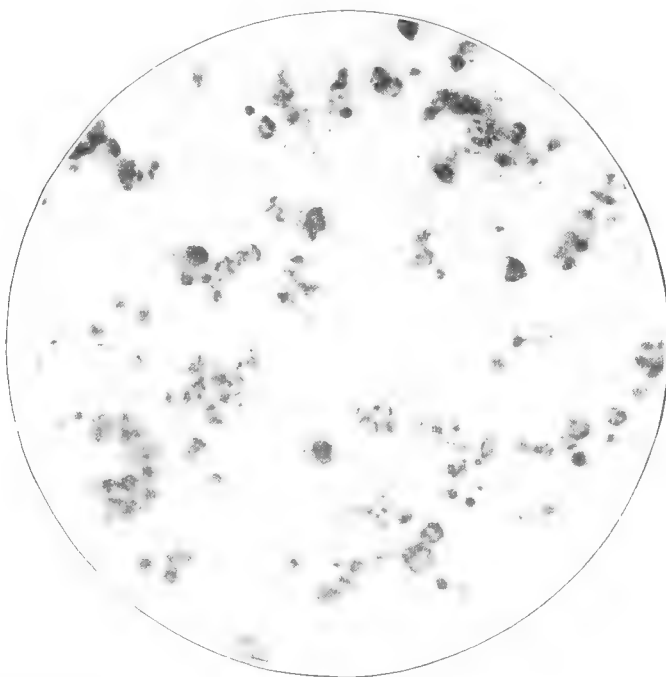


Fig. 3.—Nine days' growth in ascites phosphate broth, showing coccoid bodies with regular and irregular chromatin masses. Giemsa's stain; $\times 700$.

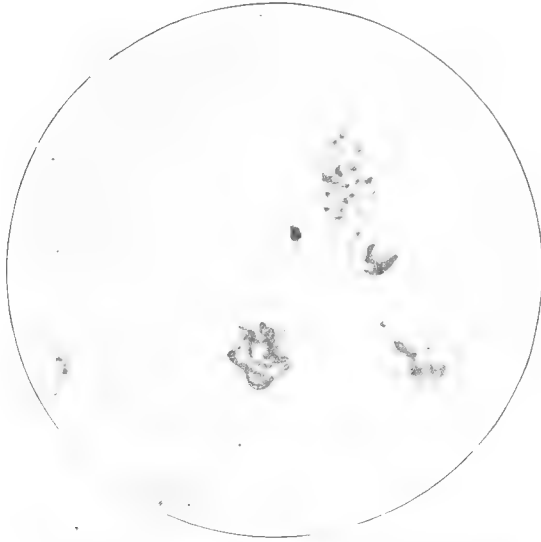


Fig. 4.—Nine days' growth in ascites phosphate broth, showing large coccoid body with chromatin arranged in coiled strands. Giemsa's stain; 1:1,200.

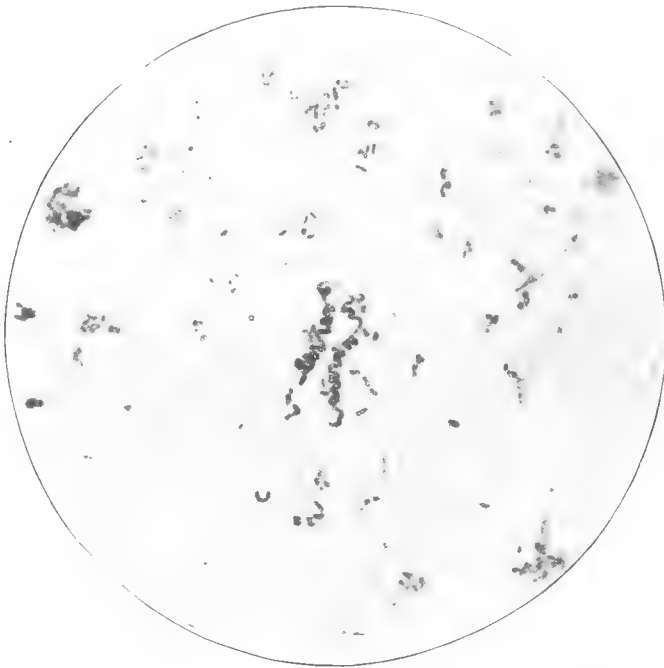


Fig. 5.—Three days' growth in ascites phosphate agar. The specimen shows short bacillary forms and the coiled strands unwinding into wavy filaments. Giemsa's stain; 1:1,200.

Herrold's⁵ dibasic sodium phosphate 1% agar was made with Bactobeeff and peptone 0.1% of dibasic sodium phosphate, adjusted to p_H 7.4-7.6.

The phosphate broth was made according to the formula used by Havens and Taylor.⁶ Meat infusion broth was made with distilled water, to which was added 1% peptone, 1% disodium phosphate and 0.5% glucose. It was adjusted to p_H 8-8.2. Tubes containing 10 c.c. of the broth were autoclaved at 10 pounds for 10 minutes. Ascites fluid was added in the proportion of 1 part of ascites fluid to 3 parts of broth.

Sterile hanging drop preparations sealed with paraffin were studied for a period of 48 hours in a warm stage microscope. Specimens containing the large round forms were seen to disintegrate and the short bacillary forms to appear, but no other stages in the development of the organisms were observed.

ANIMAL EXPERIMENTS

Eight guinea-pigs were injected intraperitoneally with from 1-2 c.c. of ascites phosphate dextrose broth. A fibrinous peritonitis was produced, the guinea-pigs dying in 2 to 3 days. Specimens of peritoneal fluid were removed at varying intervals after inoculation and also after death, and studied by dark-field illumination and in stained specimens. As a rule, only the short bacillary forms with bipolar granules were observed, but occasionally longer, thicker bacilli staining a deep blue with Giemsa, and small coccoid bodies containing deeply staining chromatin, were seen. The organism was recovered in pure culture broth before and after death and showed the same variety of forms observed in the original culture.

The inoculation of 5 c.c. of a similar culture into the pleural cavity of a rabbit produced a profuse seropurulent pleuritis with almost complete collapse of the lung, the animal dying in 5 days. Another rabbit received an intracranial inoculation of 1 c.c. An abscess formed in the subcutaneous tissue of the head at the site of inoculation which apparently inconvenienced the animal very little and finally healed. It suffered no other ill effects. Short bacillary forms were observed in smear preparations from these lesions and were isolated in pure culture in both cases.

DISCUSSION

We have been unable to find in the literature an organism which corresponds to this pleomorphic bacillus. It is possible that there may be organisms similar to it that occur in diseased conditions in only one stage. The smears from the tonsillar granules from which these organisms were isolated showed large motile bacilli, spirilla, filaments and cocci. The filamentous forms with the chromatin arranged in definite bodies

⁵ Personal communication.

⁶ Am. Jour. Hyg., 1921, 1, p. 311.

and in strands resemble those seen in the cultures. The small bacillary forms might easily be overlooked in the original material. The large coccoid bodies, which we were unable to see in the smears, may of course be the result of artificial cultivation.

The chromatin material arranged in distinct bodies, spirals, crescents, rods or filaments is perhaps nuclear in nature, according to Swellengrebel,⁷ Dobell,² Douglas and Distaso,⁸ Meyer,⁹ Löhnis⁴ and others.

The large spherical cells with large round chromatin bodies and those with the chromatin arranged in skeins or masses of threads resemble the form of *Spirochaeta pallida* described by Ross,¹⁰ Jennings,¹¹ and Molgavkar¹² in fresh syphilitic material, the chromatin within the cell inclusions becoming formed into spirochetes. The occasional formation in our cultures of spiral forms from the chromatin masses in the cell indicated that this occurred in the culture.

Our coccoid forms with the chromatin arranged irregularly or as a round mass centrally or excentrally placed, resemble the forms described by Negri¹³ as "nekrobiotischen Herde," in her cultures of mycobacteria.

Mellon¹⁴ describes in a fusiform bacillus culture giant cocci which contained distinct oval intracellular bodies, which are suggestive of the large coccoid forms in our cultures. His giant forms also contained chromatin granules with intercommunicating filaments. These cells appeared to disintegrate leaving pleomorphic granules attached to long wavy filaments. These sometimes broke up into vibrios and then into small coccus forms.

The coiled forms in our cultures resemble the rolled up filaments and balls of *Rickettsia lectularia* described by Hertig and Wolbach¹⁵ in smears from mycetome from a bed bug. This organism also is gram-negative, small and pleomorphic, with its minute coccoid and diplococcoid bodies, curved, bent and straight rods, bacillary forms and filaments.

We are indebted to Dr. E. O. Jordan for the species name and to Dr. D. H. Bergey and Dr. F. Löhnis for suggestions on classifying this organism. Dr. Bergey is inclined to place it under the genus *corynebacterium*. Because of our present incomplete knowledge of bacteria,

⁷ Ann. de l'Inst. Pasteur, 1907, 21, p. 448, 562; Arch. f. Hyg., 1909, 70, p. 380.

⁸ Centralbl. f. Bakteriolog., I, O., 1912, 63, p. 1; 1912, 66, p. 321.

⁹ Die Zella der Bakterien, 1912.

¹⁰ Brit. Med. Jour., 1912, 2, p. 1651.

¹¹ Ibid., p. 1655.

¹² Ibid., p. 1655.

¹³ Folia Microbiologica, 1916, 4, p. 119.

¹⁴ Jour. Bacteriol., 1919, 4, p. 505.

¹⁵ Jour. Med. Res., 1924, 44, p. 327.

Dr. Löhnis considers it simpler to designate it as bacillus. Since our organism does not fully correspond with the characteristic of either corynebacterium or the influenza group, which it also resembles, we have decided tentatively to classify it a bacillus.

SUMMARY

A small, gram-negative, pleomorphic, anaerobic organism was isolated from a tonsillar granule. During the course of its development, it appears as bacilli, large and small coccoid forms, straight and wavy filaments. None of these forms is motile. The organism multiplies by fission, budding and by the production of gonidangia and gonidia. The different forms contain chromatin material arranged in a great variety of shapes.

CAPSULATED BACTERIA

WITH SPECIAL REFERENCE TO *B. TYPHOSUS*

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There is little definite knowledge concerning capsules and their rôle in the activities of bacteria. Most of the investigations have been made with reference to the development of staining methods, and we find nearly as many procedures as there are investigators. A few bacteria are commonly known to develop capsules—especially under specific cultural conditions—and simple stains will demonstrate them. Some organisms, generally considered noncapsulated, have been shown to possess capsules when studied under special conditions.

In 1896, Migula¹ stated that all bacteria possessed capsules, but that only a few could be stained. Strong,² in 1899, reached the following conclusions: "Artifacts occur with such frequency that it is quite easy to mistake them for capsules. A hazy corona may not indicate a capsule. The capsules are composed of a gelatinous substance formed from the outer layers of the cell membrane, which take up water and are partly dissolved. The replacement is by the active growth of the inner layers. Capsules with definite membranes occur only in tissues and exudates." Buerger³ reported capsules on the pneumococcus, *B. lactis aerogenes*, and streptococcus pyogenes. Also, he found faint capsules on *B. coli*, *M. catarrhalis*, the gonococcus, the meningococcus, *B. diphtheriae*, and the staphylococcus. Kayser⁴ demonstrated capsules on Friedländer bacilli and on anthrax bacilli. He failed to obtain them with *B. coli* and *B. pyocyaneus*. Buerger⁵ studied the capsules of pneumococci and streptococci. He found what he termed certain regularly defined types of capsules. Eisenberg⁶ believed that capsule formation by *B. anthracis* was due to a chemical reaction between the ectoplasm of the bacterial cell and some unknown albumin-like substance in animal serum. Later, he concluded that "highly virulent bacteria surround themselves in the

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¹ Deutsch. thierärztl. Wchnschr., 1896, 4, p. 28.

² Centralbl. f. Bakteriöl., I, 1899, 25, p. 49-52.

³ Ibid., I, O., 1905, 39, pp. 216, 335.

⁴ Ibid., I, O., 1906, 41, p. 138.

⁵ J. Infect Dis., 1907, 4, p. 426.

⁶ Centralbl. f. Bakteriöl., I, O., 1908, 47, p. 415.

infected animal host with a thick capsule which is lost when the bacteria are transferred to artificial media." Kuwabara⁷ reported a capsulated organism from a case of ophthalmia. At first, it was thought to be the Friedländer bacillus, but later he found the cultural characteristics conformed to those of *B. coli*. Nunokawa⁸ inoculated animals with *B. anthracis* and studied the morphologic changes which occurred in the the bacteria. The organisms were also grown in the presence of blood. He concluded that the same capsular changes occurred in the animal body and in the presence of blood serum in vitro. Fürst⁹ postulated that capsules constituted no essential part of bacteria. Kühnemann¹⁰ experimented with diluted serums, and determined that capsule formation was most pronounced in dilutions from 1:5 to 1:10. His explanation was that blood contains an irritating stimulant (antibacterial bodies) and that as a result of the action of this stimulus the bacterial cell surrounds itself with a capsule as a means of self defense. Rosenow¹¹ found that capsules of the pneumococci and allied organisms were not difficult to preserve nor readily soluble in water. Staining was a problem of rendering the capsule stainable rather than of preserving it. Rosenow believed that the pneumococcus capsule was not a factor in virulence. Carpano¹² maintained that capsular substance was produced from the medium by a biochemical process induced by the bacteria, and that this substance became attached to the cell in the form of a capsule. He regarded a capsule as "nothing other than a constant structural peculiarity" which it was not always possible to recognize because of imperfect technic. He claimed to have demonstrated capsules in 24-hour agar cultures of *B. typhosus*, *B. suipestifer* and *B. mallei*.

Marrassini¹³ described a capsulated typhoid bacillus and believed that a covering, varying in thickness, surrounded the cells of *B. typhosus*, *B. subtilis*, the cholera vibrio, *Staphylococcus aureus* and *B. anthracis*. Gózonyi,¹⁴ using an India ink stain, studied capsule formation by the organisms of the hemorrhagic septicemia group and concluded that capsules were as characteristic of these organisms as bipolar staining. Uémura¹⁵ reported capsules on anthrax bacilli grown in serum mediums.

⁷ Arch. f. Augenh., 1908, 60, p. 323.

⁸ Centralbl. f. Bakteriol., I, O., 1909, 53, p. 317.

⁹ Ibid., I, O., 1910, 56, p. 97.

¹⁰ Ibid., I, O., 1911, 57, p. 497.

¹¹ J. Infect. Dis., 1911, 9, p. 1.

¹² Centralbl. f. Bakteriol., I, O., 1913, 70, p. 42.

¹³ Ibid., I, O., 1913, 71, p. 113.

¹⁴ Ibid., I, O., 1913, 68, p. 594.

¹⁵ Ibid., I, O., 1914-15, 75, p. 21.

Huntoon¹⁶ used a medium containing nutrose, and modified the staining technic by discarding heat for fixing the preparations and employing a weak solution of lactic acid as a precipitating agent. Fletcher¹⁷ isolated mucoid colonies of *B. paratyphosus* B and believed that the cells from these colonies were capsulated because of their large silhouettes, but he was unable to obtain stained preparations. He also studied mucoid colonies derived from cultures of *B. dysenteriae*, but his results are not convincing.

New Methylene Blue Capsule Stain 1.—Since the question of the presence of a capsule on the typhoid bacillus is still open, this study was made primarily for the purpose of obtaining more exact information on this point. Not only must the stain be considered, but also other factors such as the medium and the time of incubation of the cultures. Tests were first made with a strain of the pneumococcus which regularly gave good stains by the ordinary methods when the organisms were grown in milk. Numerous other mediums were used, but the most satisfactory results were obtained with a dextrose-meat-infusion broth containing one-tenth part each of blood serum and ascitic fluid. Previous observations had indicated that an acid solution of methylene blue would stain capsules. The following method proved satisfactory with the test organism.

1. Fix smear and apply glacial acetic acid for 10 to 15 seconds.
2. Stain for 2 minutes with a mixture containing 2.5 c.c. of a saturated alcoholic solution of methylene blue in 25 c.c. of 5% aqueous phenol.
3. Wash off the stain gently with a fine stream of distilled water.
4. Dry and examine.

The organism appeared black in the center of a blue capsule which surrounded the cell uniformly (Fig. 1).

This stain was then tested on several organisms, Welch's stain being used as a check. Milk cultures were used. Capsules were demonstrated on *B. lactis aerogenes*, *B. mucosus capsulatus*, *B. rhinoscleromatis*, *B. welchii*, *B. avisepticus*, *B. bovissepticus*, the bacillus of slimy milk and several strains of streptococci. All the preparations compared favorably with those made by Welch's method and in some cases were superior. Better results were obtained after 48 hours' incubation than after 24 hours'. With some organisms, capsules could be demonstrated during 10 days' incubation. The stain has given satisfactory results in the hands of students.

An attempt was made to develop demonstrable capsules on *B. typhosus* by culturing in the presence of rabbit serum. One c.c. quantities of a number of dilutions of rabbit serum (1:1, 1:2, 1:6, 1:12, 1:25, 1:50) were added to 24-hour cultures of *B. typhosus*. Capsule stains were made after 12 hours' incubation at 37 C. The results were negative with the stains of Welch, Rosenow, Hiss, Carpano and methylene blue stain 1 just described. The cultures were incubated for 24 hours, centrifuged at high speed and the cells washed 3 times with physiologic salt solution. Again, the stains failed to demonstrate capsules.

¹⁶ J. Bacteriol., 1917, 2, p. 241.

¹⁷ Lancet, 1918, 2, p. 102.

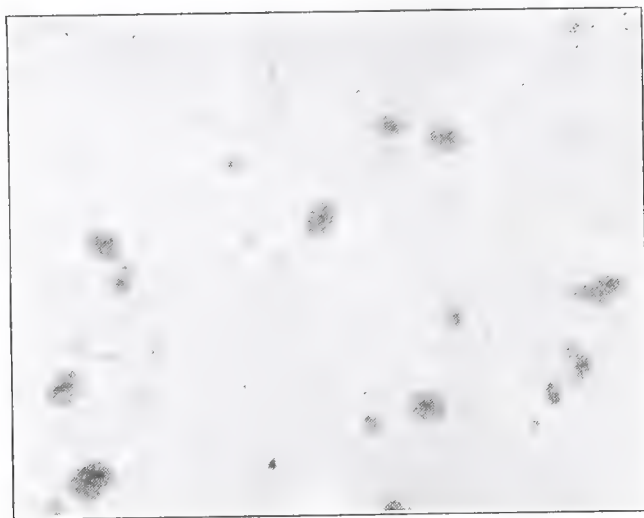


Fig. 1.—*B. mucosus-capsulatus*, showing capsules stained with methylene blue capsule stain 1.

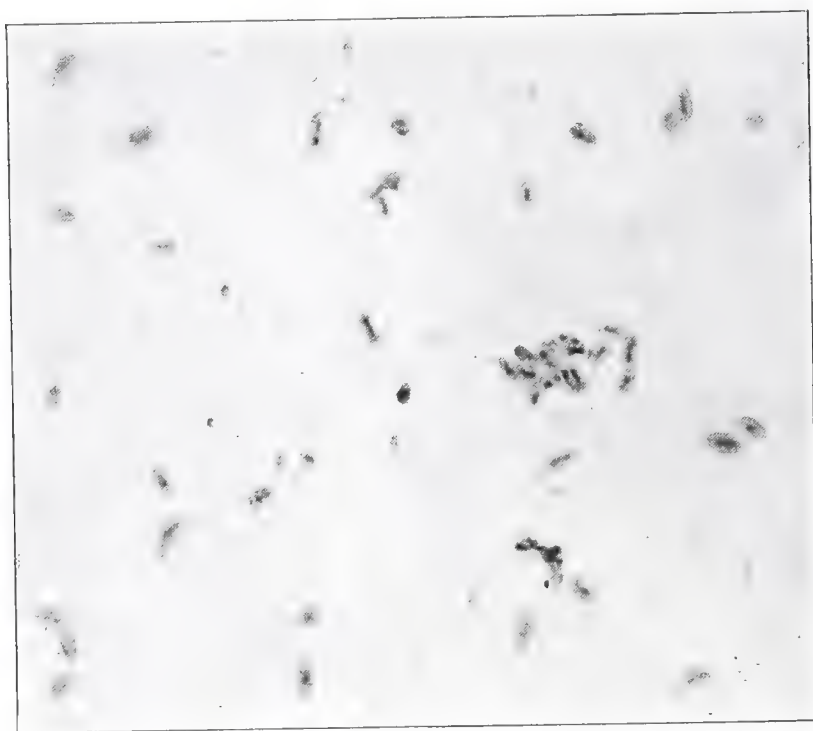


Fig. 2.—Capsules of *B. typhosus* stained with methylene blue capsule stain 2.

Methylene Blue Capsule Stain 2.—A variation of stain 1 was now developed, and with this second stain pronounced capsules were demonstrated on cells of *B. typhosus* (Fig. 2). The technic was as follows:

1. Prepare smear in the usual way and fix by passing the slide rapidly through the flame 2 or 3 times.
2. Flood the slide with glacial acetic acid for not more than 10 seconds. The time will depend on the thickness of the smear. When the smear is visible but appears about to vanish the acetic acid should be removed.
3. Remove the acid by washing 3 or 4 times with the methylene blue stain prepared as follows: Dissolve 5 gm. of tannic acid in 50 c.c. of a 5% solution of phenol. When solution is complete, add 10% by volume of a saturated alcoholic solution of methylene blue and mix thoroughly.
4. Place the slide over a steam bath and add one half as many drops of a 1% solution of KOH as there are drops of stain on the slide.
5. Steam gently for 10 minutes.
6. Wash off stain with a fine stream of water from a wash bottle. The capsules appear uniformly stained blue surrounding the cells which are stained black.

With this technic capsules have been demonstrated on 3 strains of *B. typhosus*, on *B. coli*, *B. enteritidis*, *B. dysenteriae*-Shiga, *B. paratyphosus* A, *B. paratyphosus* B, *B. fecalis-alkaligenes*, *B. proteus-vulgaris* and *B. cloacae*. The organisms were grown in the presence of serum as described above. When the serum dilution was from 1:3 to 1:18, capsules were obtained. At all other dilutions the results were negative. Capsules were readily demonstrated during the first 7 days' incubation, but all results were negative after this period.

The relation between motility and capsule formation was then studied. It was found that at the time capsules were demonstrable, the cells had lost their motility.

SUMMARY

Capsules have been demonstrated on cells of *B. typhosus*, *B. coli*, *B. enteritidis*, *B. dysenteriae*-Shiga, *B. paratyphosus* A, *B. paratyphosus* B, *B. fecalis-alkaligenes*, *B. proteus-vulgaris*, and *B. cloacae*.

The organisms which are normally motile were found to be nonmotile during the period when capsules could be demonstrated.

Two methylene blue capsule stains have been developed. The first one is comparatively simple and is as satisfactory as Welch's stain for the commonly encapsulated organisms. The second stain is somewhat complicated, but in our hands has been successfully used to demonstrate capsules on nine organisms not generally considered as possessing capsules.

STUDIES ON THE ETIOLOGY OF ACUTE RHEUMATIC FEVER

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Since 1899, when Westphal, Wassermann and Malkoff¹ isolated a streptococcus from the blood of a patient having chorea and endocarditis, there have been discussions in favor of and against the belief that the streptococcus or diplococcus is the etiologic agent of acute rheumatic fever and its associated pathologic changes. An apparent agreement that acute rheumatic fever is an infectious disease has evolved, but opinion is still divided as to whether the disease is of streptococcal origin or whether it is produced by an unknown virus.

The chief points of the problem to be considered seem to be: 1. Can the organism be uniformly isolated from the blood, joints, and pericardial exudates in cases of acute rheumatic fever and chorea? 2. What are these organisms? 3. Do they represent a specific strain or a heterogeneous group? 4. Are antibodies against these organisms found in the blood of patients from whom the organisms are isolated, 5. Can lesions be produced in animals similar to rheumatic lesions in human cases? With these questions in mind, the present work was undertaken.

BACTERIOLOGY OF ACUTE RHEUMATIC FEVER

A diplococcus or streptococcus has on various occasions been isolated from the blood, the joints, or the pericardial exudate in cases of acute rheumatic fever by these workers: Triboulet and Coton,² Westphal, Wassermann and Malkoff,¹ Poynton and Paine,³ Beaton and Walker,⁴ Walker and Ryffel,⁵ Loeb,⁶ Camisa,⁷ Beattie and Yates,⁸ Collins,⁹ Rosenow,¹⁰ LaFetra,¹¹ Swift and Kinsella,¹² Quigley,¹³ and others. Swift and Kinsella¹² regularly failed

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¹ Berl. klin. Wchnschr., 1899, 36, p. 638.

² Compt. rend. Soc. biol., 1898, 50, p. 124.

³ Lancet, 1900, 2, p. 861.

⁴ Brit. Med. Jour., 1903, 1, p. 237.

⁵ Brit. Med. Jour., 1903, 2, p. 659.

⁶ Arch. Int. Med., 1908, 2, p. 266.

⁷ Centralbl. f. Bakteriologie, 1910-11, 57, p. 99.

⁸ Jour. Path. & Bacteriol., 1912-13, 17, p. 538.

⁹ Brit. Med. Jour., 1903, 1, p. 220.

¹⁰ Jour. Infect. Dis., 1914, 14, p. 61.

¹¹ Arch. Pediat., 1915, 32, p. 135.

¹² Arch. Int. Med., 1917, 19, p. 381.

¹³ Jour. Infect. Dis., 1918, 22, p. 198.

to isolate organisms from inflamed joints. On the other hand, there are those who in repeated attempts have failed to find any organisms in the blood or exudates of rheumatic or choreic patients (Philipp,¹⁴ Cole,¹⁵ etc.)

The organisms discussed in this paper were isolated in 20 cases of well defined, acute rheumatic fever, rheumatic endocarditis or chorea. Five of these cases came to necropsy. All of the 20 strains were isolated from the blood before death except 7. Two of the 7 were isolated from rheumatic joints before death, 2 from the pericardial exudates at necropsy, 1 from the pericardial exudate and spleen at necropsy, and 2 from the blood at necropsy. Three additional organisms from cases of endocarditis corresponding to subacute bacterial endocarditis (two checked by necropsy and one a typical clinical case of subacute bacterial endocarditis) were compared with the rheumatic strains morphologically, culturally, immunologically, and in their ability to produce experimental endocarditis.

There are several clinical characteristics common among the 20 rheumatic cases from which organisms were isolated. The disease occurred early in life, in the second and third decades, except in one case. This was a baby only a few days old. The mother developed acute rheumatic fever a short time before delivery. Similar organisms were isolated from the baby's joints and the mother's blood. The joints were involved in all of the 20 cases and the hearts in all but four. Chorea occurred once. The temperature seldom went above 102 F. The blood picture is of especial interest. The hemoglobin did not drop below 80 per cent. This is significant when compared with the low hemoglobin content of cases of subacute bacterial endocarditis. The red blood cells were usually nearly normal in number. There was usually a fair degree of leukocytosis, average about 15,000. In these 20 cases, death occurred in 6. Necropsies were obtained in 5.

The following is a representative case: A young white man, aged 18, admitted to the hospital, May 1, 1924, complained of pains in the joints, dyspnea, rapid and forceful heart beat, and precordial pain. He became sick, April 4, 1924, with fever and chills. The ankles, knees, wrists and shoulders became swollen, red and painful. In the third week, the pains in the joints subsided but dyspnea increased. The precordial pain continued to grow worse. At 8 years of age, he had had tonsillitis followed by acute rheumatic fever which lasted 5 months. His tonsils were removed at the age of 10. Physical examination on admission showed the heart to be enlarged to both left and right. A loud to and fro murmur at the aortic area replaced the valve sound. At the mitral area, the first sound was indistinct, and a loud diastolic murmur was heard. The temperature varied from 99.5 to 102 F. The blood showed 80 per cent. hemo-

¹⁴ Deutsch, Arch. f. klin. Med., 1903, 76, p. 150.

¹⁵ Jour. Infect. Dis., 1904, 1, p. 714.

globin, 4,000,000 red cells and 20,000 leukocytes. The roentgen ray showed a greatly dilated heart with some increased fluid in the pericardial cavity. *Streptococcus viridans* was grown from the blood. The patient improved and was discharged, July 23, 1924.

Technic. Fifty cc. of blood was the usual amount taken for cultural purposes. This is important when there are but few organisms in the blood, as is usually the case in acute rheumatic fever. The blood was collected in 2 large sterile test tubes and allowed to clot. These clots when firm were loosened and put into flasks containing 250 c c. of dextrose (0.2%) beef infusion broth with a reaction of P_H 7.6. The firm clot seems to favor a partial anaerobic culture, and at the same time any antibodies that may be present are diluted in the fluid medium. The cultures were incubated at 37 C. for several days. Rarely was the organism found in the cultures by smear in less than 5 days. The usual time was about 10 days after the beginning of incubation. In one case, the organism was recovered after incubating for nearly a month. It is evident that a higher percentage of positive cultures would be found if examinations were made over longer periods of time. The streptococci frequently grow slowly when isolated. When there are but few organisms in the blood, some time may be required before they can be detected in 250 c c. of broth by smear. As soon as the organisms were found in the original cultures by smear, they were plated on blood agar from which colonies were picked. All work with animals was carried on with pure cultures. The animals were injected with large doses 3 times a week. The strains were studied morphologically, culturally, immunologically and by animal experimentation.

NATURE OF THE ORGANISMS

Most workers who have isolated organisms from rheumatic patients report diplococci or streptococci. Poynton and Paine¹⁶ called their organism a diplococcus. The action on the blood-agar plate is not well described in the older literature. Beaton and Walker⁴ and Walker and Ryffel⁵ have described their strains as producing a brownish discoloration. Lyall¹⁷ found his strains to be inactive on blood. Rosenow¹⁰ reported strains some of which were inactive on blood, some produced green, while others were hemolytic. Swift and Kinsella¹² found that their organisms belonged in the viridans group. They believe that the streptococci described by earlier workers correspond to what is now known as *Streptococcus viridans*.

¹⁶ Lancet, 1910, 1, p. 1524.

¹⁷ Jour. Med. Res., 1914, 30, p. 487.

The biologic tests, as reported in the literature, have shown that not all organisms isolated from rheumatic patients belong to the same group. Andrewes and Horder¹⁸ found that one of Poynton and Paine's strains belonged to the buccalis group and one of Beattie's to the fecalis group. Beattie and Yates¹⁹ reported 3 strains isolated from knee joints as belonging in the fecalis group. Lyall¹⁷ reported the fermentation reactions of 5 organisms isolated from rheumatic patients. Three of the 5 fermented mannite and were grouped as fecalis. Quigley¹³ examined 6 strains, 4 of which fermented mannite. The organisms reported in the literature are usually streptococci, nonhemolytic in character.

Most of my strains when first isolated are chiefly arranged as diplococci. They differ from such diplococci as the gonococcus and the meningococcus in that the individual cocci are elongated in the long axis of the chain. They have a tendency to be lance-shaped and in this resemble the pneumococcus. They differ from the pneumococcus in not being bile soluble, in not fermenting inulin, and in not being capsule producers. It cannot be said that these strains never grow out into chains, but the marked tendency is for them to appear as diplococci. It is unusual to find spontaneous agglutination in broth cultures.

The inability to produce a green discoloration on the blood-agar plate at 24 hours' incubation is an almost uniform characteristic of my strains when first isolated. I have never found this characteristic in any other group of streptococci except the fecalis group isolated from feces and commercial milk starters. All the inactive strains from rheumatic fever cases, after a period of cultivation, became producers of green on the blood-agar plate. The percentage of mannite fermenters is high. This places these strains in the fecalis group, which is in accord with the findings of others.

One strain is a typical beta hemolyzer. This strain was found in pure culture in relatively large numbers in the pericardial exudate of a child dying during an attack of acute rheumatic fever with pericarditis. Aschoff nodules were found in the myocardium. This is in agreement with the findings of Rosenow.¹⁰ Jackson²⁰ reported the production of typical rheumatic lesions in the myocardium of rabbits by the injection of hemolytic streptococci.

From table 1, it is apparent that at least 2 groups of organisms are represented by agglutination (dilutions 1:10,000). Fairly uniform

¹⁸ Lancet, 1906, 2, pp. 178 and 775.

¹⁹ Jour. Path. & Bacteriol., 1911-12, 16, p. 247.

²⁰ Jour. Infect. Dis., 1912, 11, p. 243.

agglutination results were obtained between serums 2, 6, 13, 9, 5, 17 and 1 and the 14 organisms tested. Eleven of these organisms were of rheumatic origin, and 3 were from cases of subacute bacterial endocarditis. The first 7 serums may be considered as representing a homogeneous group. No agglutination occurred with serums 4, 8 and 11, except with their homologous strains. These 3 strains may be considered as representatives of a heterogeneous group. Three serums produced by organisms isolated from cases of subacute bacterial endocarditis were tested with the same 14 organisms. Two of these serums fell into the homogeneous group and one into the heterogeneous group. Agglutination apparently does not differentiate strains of rheumatic origin from strains of subacute bacterial origin. These agglutination reactions are similar to the agglutination reactions of streptococci from other sources. An interesting relation appears between the biologic and immunologic characteristics. All strains in the homogeneous group are mannite fermenters, while those in the heterogeneous group fail to ferment mannite.

AGGLUTINATIONS 1:10,000

Serums against Rheumatic Strains	Organisms													
	Rheumatic Origin											Subacute Bac- terial Origin		
	1	2	4	5	6	8	9	11	13	16	17	7	15	19
2.....	+	+	+	+	+	—	+	+	+	+	+	+	+	—
6.....	+	+	+	+	+	—	+	—	+	+	+	+	+	—
13.....	+	+	—	+	+	+	+	—	+	+	+	+	+	+
9.....	+	+	—	+	+	+	—	—	+	—	+	+	+	—
5.....	+	+	+	+	+	—	+	—	+	—	+	+	+	—
17.....	+	+	—	+	—	—	+	—	+	—	+	+	+	—
1.....	+	+	—	+	—	—	+	—	+	+	+	+	+	—
4.....	—	—	+	—	—	—	—	—	—	—	—	—	—	—
8.....	—	—	—	—	—	—	—	—	—	—	—	—	—	—
11.....	—	—	—	—	—	—	—	+	—	—	—	—	—	—
Serums against Subacute Bacterial Endocarditis Strains														
19.....	—	—	—	—	—	—	—	—	—	—	—	—	—	+
7.....	+	+	+	+	+	+	+	—	+	+	+	+	+	—
15.....	+	+	+	—	+	—	—	+	+	—	+	+	+	—

SPECIFICITY OF THE ORGANISMS

Poynton and Paine³ and others consider the streptococcus isolated by them in cases of acute rheumatic fever and chorea to be a specific strain. Walker and Ryffel⁵ have differentiated their strains from other strains of streptococci by their ability to grow in the filtrate of cultures of typical strains of streptococci (the Mamorek test). Beattie and Yates,⁸ on the basis of the biologic tests and pathogenesis, state that the strepto-

cocci isolated from rheumatic patients cannot be differentiated from other strains of streptococci. Swift and Kinsella¹² failed to find uniformity among their strains by agglutination and complement-fixation tests as well as by the biologic tests.

The fact that various workers have been able to produce multiple arthritis and endocarditis in animals by injecting organisms isolated from rheumatic cases has apparently failed to confirm the idea of the specificity of these organisms. Cole,¹⁵ Davis,²¹ Henrici,²² and others have shown that endocarditis and nonsuppurative arthritis can be produced in rabbits by injecting streptococci, hemolytic or viridans, from various sources other than rheumatic fever or endocarditis.

The conclusion to be drawn from the findings of various workers and the findings with my strains on the basis of morphologic, cultural, and immunologic characteristics is that the group cannot be considered a specific one, but that it represents a heterogeneous group, generally green producers with moderately low virulence.

IMMUNE BODIES IN PATIENTS' SERUM

Relatively little work has been done to determine the incidence of immune bodies in the serums of rheumatic fever patients. Tunncliffe²³ found that opsonins for streptococci were increased in the blood of patients having acute rheumatic fever. In the serum in 7 cases of 12, she found agglutinins for the same organisms. Kinsella²⁴ found in testing the blood of 12 patients having subacute bacterial endocarditis that all showed the presence of agglutinins. He used no dilutions over 1:40. In 5 patients having acute rheumatic fever, Swift and Kinsella¹² were unable to find agglutinins. I have tested 5 cases. In 4, agglutinins were found in a dilution of 1:50 or more. In one, they could not be detected. The serum of 3 rheumatic patients agglutinated a streptococcus isolated before death in a case of subacute bacterial endocarditis. This case was found to be typical at necropsy.

ANIMAL EXPERIMENTS

In considering whether the streptococcus may be the etiologic agent in producing acute rheumatic fever, much importance rests on whether lesions similar to human rheumatism lesions can be produced experimentally in animals.

²¹ *Ibid.*, 1912, 10, p. 148.

²² *Ibid.*, 1916, 19, p. 572.

²³ *Ibid.*, 1909, 6, p. 346.

²⁴ *Arch. Int. Med.*, 1917, 19, p. 367.

Many investigators have produced endocarditis in rabbits and a few in dogs and monkeys. On this there is general agreement, but the point on which workers fail to agree is whether the endocarditis is structurally similar to that found in human rheumatic cases. Poynton and Paine¹⁶ believe that the same organism may produce either simple (rheumatic) endocarditis or malignant (subacute bacterial) endocarditis. Beattie and Dickson²⁵ report experimental data to show that an organism isolated in a case of subacute bacterial endocarditis may when injected into one animal produce a simple endocarditis and when injected into another animal, a malignant endocarditis. The work of Cole,¹⁵ Davis,²¹ Jackson,²⁰ and Henrici²² shows that streptococci from other sources are capable of producing endocarditis.

The microscopic myocardial lesions seem to be the most difficult to reproduce. Thalheimer and Rothschild²⁶ have produced nodular lesions in the myocardium of rabbits by injecting streptococci, but they do not consider these lesions similar to the typical Aschoff nodule. They think their lesions and the lesions of Bracht and Wächter²⁷ have neither the structure, location, typical cells nor staining reaction characteristic of the Aschoff nodule. On the other hand, Coombs, Miller and Kettle,²³ Jackson,²⁰ and Rosenow¹⁰ report that they have produced the Aschoff nodules in the myocardium of rabbits, and that these nodules are typical in all respects of those found in man in acute rheumatic fever.

The gross rheumatic valvular lesion in man consists of small smooth globular masses on the free margins and at the points of contact of the valves. The microscopic lesion consists of proliferative and exudative inflammation which begins within the valve proper, often some distance beneath the endothelium. The proliferation may be moderate or pronounced. The exudation consists of fibrin and cells, mostly large mononuclear and multinuclear cells, though small lymphocytes and a few polymorphonuclear cells are generally present. There is usually a necrotic center in the nodule of inflammation. This necrosis may be found in a degree which can scarcely be noticed or in so extensive a degree that the center of the nodular mass on a valve may have undergone complete necrosis. The endothelium usually ruptures, and a small thrombotic mass from the blood content may collect. The inflammation in a cardiac valve is essentially that of the Aschoff nodule or of a subcutaneous nodule. In the valve, as in the subcutaneous nodule, the

²⁵ Special Pathology, Ed. 2, 2, p. 507.

²⁶ Jour. Exper. Med., 1914, 19, p. 429.

²⁷ Deutsch. Arch. f. klin. Med., 1909, 96, p. 493.

²⁸ Lancet, 1912, 2, p. 1209.

nodules of inflammation are multiple. This microscopic picture of rheumatic inflammation is for the most part similar to that described by Swift²⁹ in his study of rheumatic lesions in the heart, joints, and subcutaneous nodules. With these conditions in mind, the lesions of rabbits were studied to determine whether a similarity existed.

Twelve of the 20 strains reported in this series were tested in rabbits. Nine produced endocarditis. Strain 1 was injected into 4 rabbits. In 2 of these, typical endocarditis developed. In one, there was an early endocarditis; in the other, a well defined mitral and aortic endocarditis. The vegetations have a smooth nodular appearance resembling rheumatic endocarditis in human cases.

Four rabbits were inoculated with strain 2 without the development of any endocarditis. Two rabbits were inoculated with strain 3. There was endocarditis in one.

Five rabbits were injected with strain 4. No endocarditis developed in any of these rabbits. Three of the 5 died within 48 hours with septicemia. In one which received injections over a period of 2 months, there was a well defined arthritis. The joints were red, swollen and tender. These joints were needled, and a serous fluid was obtained from which organisms similar to those injected were recovered in large number in pure culture.

Four rabbits were used with strain 5, and well defined endocarditis developed in 2. The nodules in the cusps were smooth, hard and white (fig. 1).

Four rabbits received injections of strain 6. A well defined tricuspid endocarditis with smooth glistening nodular vegetations was produced in one.

Strain 8 was injected into 3 rabbits. Endocarditis (fig. 2) occurred in 2. In one, an extreme fibrinous pericarditis developed. The cellular exudate in this pericarditis was similar to that found in human rheumatic pericarditis in being chiefly mononuclear.

Two rabbits received injections of strain 9. In one, several small smooth nodular lesions developed on the mitral cusps. In the other, there was an extreme stenosis of the aortic orifice. This rabbit died a typical cardiac death with extreme passive congestion of the liver. An infarct was present in the kidney. The valvular nodules were large, white and smooth. Extreme polyarthritis with swollen red and tender joints developed. A needle was inserted into one of these joints, and

²⁹ Jour. Exper. Med., 1924, 39, p. 497.

a mucoid fluid was obtained. Organisms in pure culture in large numbers were grown from the fluid. These organisms in every way resembled those injected into the animal.



Fig. 1.—Vegetations on mitral cusps.

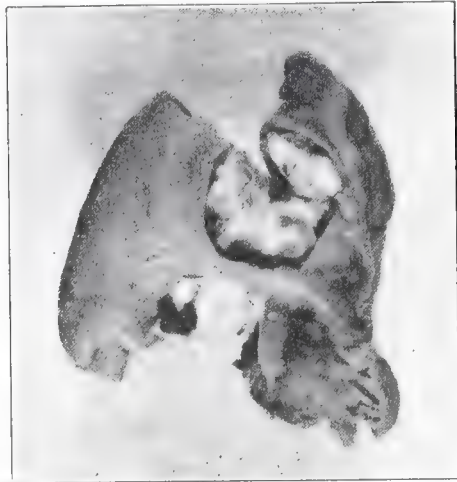


Fig. 2.—Aortic stenosis.

Three animals received injections of strain 11. In one, a moderate degree of tricuspid endocarditis appeared. The nodules on these cusps were similar to those described in the previous cases.

Two rabbits received injections with strain 13, and in one case, a well defined mitral endocarditis with stenosis occurred. The vegetations were made up of smooth glistening nodules. No endocarditis occurred in the one rabbit that received injections of strain 16.

One animal received injections of strain 17, and extreme mitral and aortic endocarditis developed. There was an almost complete stenosis of the aortic orifice.

Nine of the 12 strains (75%) produced endocarditis in rabbits with lesions grossly similar to those found in typical human rheumatic endocarditis. In all, 35 rabbits received injections with 12 strains, and 13



Fig. 3.—Mitral stenosis.

cases (37%) of endocarditis developed. The percentage would be higher if the rabbits which died in from 24 to 48 hours from septicemia were deducted. It is of interest to note that strain 15 isolated before death from a typical case of subacute bacterial endocarditis (proved by necropsy) produced an endocarditis in the one animal that received an injection. Two other strains from cases of subacute bacterial endocarditis were injected into animals without producing lesions. The lesion in the animal produced by strain 15 grossly and microscopically is similar to those produced by organisms isolated from rheumatic cases (fig. 3). This is in agreement with the work of Cole¹⁵ in which he produced endocarditis in rabbits by injecting streptococci from various sources and also with the work of Beattie and Dickson²⁵ in which they

produced malignant lesions in one animal and simple lesions in the second animal by injecting streptococci isolated from a case of subacute bacterial endocarditis.

The work with these 12 strains isolated in cases of acute rheumatic fever and the one strain isolated in a case of subacute bacterial endocarditis agrees with what many investigators have shown, namely, that lesions grossly similar to human rheumatic endocarditis can be produced in rabbits by injecting streptococci.

The microscopic appearance of the valvular lesions is similar in all the cases and also similar to the lesions studied in several human cases. The lesion seems to begin within the valve with proliferative and exudative inflammation. Large irregular mononuclear cells with vesicular nuclei are common in the cellular exudate. Multinucleated cells of the same type are frequently found. Edema of the tissues in the early lesions is pronounced. Necrosis usually occurs in the nodular lesion and extends outward and generally destroys the endothelium. Thrombosis in a slight degree occurs in practically all cases. In one case of strain 9, it approached the degree which is commonly found in cases of subacute bacterial endocarditis. This rabbit had infarcts in the kidney. In the other positive case of strain 9, the lesion was small and corresponded to the histologic findings in human rheumatic valves.

Myocarditis was present in all cases in which there was endocarditis except in 3. The myocardial lesions consisted of diffuse and nodular proliferative lesions with a slight degree of exudation. In some areas, a small amount of necrosis was present. The lesions contained large mononuclear and multinucleated cells with vesicular nuclei. Some of the nodular lesions were similar morphologically in all respects to the Aschoff nodules in human cases of rheumatic carditis (fig. 4). The lesions were essentially the same as the human rheumatic lesions.

RELATION OF RHEUMATIC ENDOCARDITIS TO SUBACUTE BACTERIAL ENDOCARDITIS

It is often difficult to distinguish clinically cases of rheumatic and subacute bacterial endocarditis when first seen. With such cases, a custom has apparently come into use to call those with a positive blood culture subacute bacterial endocarditis. Doubtful cases with positive blood cultures have been described as mild cases of subacute bacterial endocarditis, by Duncan, Oille and Detweiler.³⁰ Of Capps'³¹ 57 cases

³⁰ Jour. Am. Med. Assn., 1924, 82, p. 1721.

³¹ Ibid., 1924, 82, p. 1722.

of endocarditis with positive blood cultures, 26 were living and well after 2 years. These doubtful cases do not run the course of a subacute bacterial endocarditis but are diagnosed as such on the basis of a positive blood culture. Miller³¹ thinks that formerly such cases would have been diagnosed rheumatic endocarditis.

When the bacteriologic findings of the past and the present investigators are considered, it seems unsafe to depend entirely on bacteriologic findings for a differential diagnosis of rheumatic and subacute bacterial

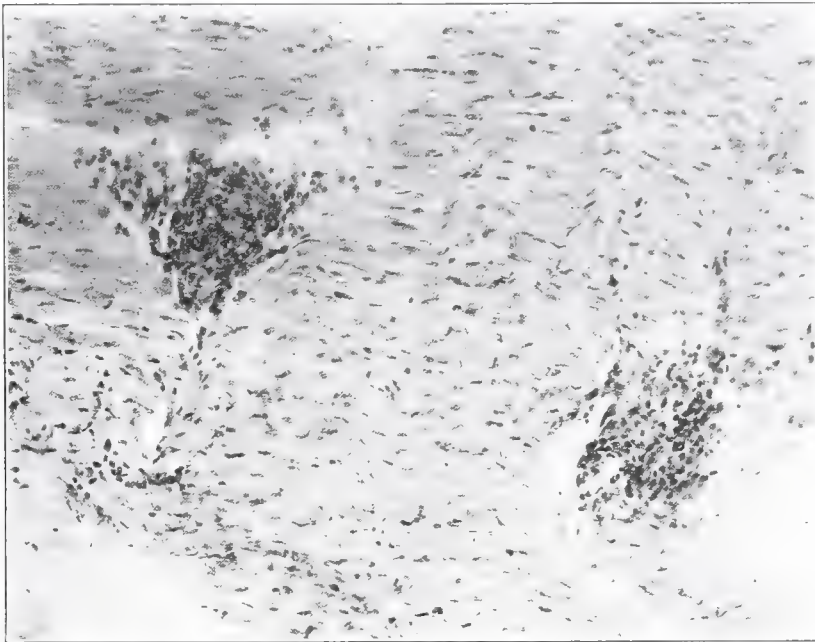


Fig. 4—Aschoff nodules in the myocardium.

endocarditis. It would appear that the two conditions may be different degrees of the same process and that subacute bacterial endocarditis may be the stage in which there is a development to the extent of an infected thrombus, while in rheumatic endocarditis there is only a stage of mild valvulitis.

SUMMARY

In a relatively high percentage of cases of acute rheumatic fever, a streptococcus can be isolated from the blood.

This streptococcus generally belongs to the viridans group. It does not seem to be a specific strain.

Lesions similar to those occurring in human rheumatic lesions can be produced experimentally in animals by injecting these organisms.

When these facts are considered, there seems to be ground for the belief that rheumatic fever with its associated lesions is produced by streptococci.

A positive blood culture in liquid medium should not form a positive basis for a diagnosis of subacute bacterial endocarditis.

There is a strong suggestion that rheumatic endocarditis and subacute bacterial endocarditis may be produced by the same agent and are but different degrees of the same process.

GROWTH OF *B. BOTULINUS* IN 30% PEPTONE

XXVII. WITH NOTES ON VAN SLYKE'S AMINO NITROGEN
METHOD FOR STUDY OF BACTERIAL METABOLISM

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A study of the chemical nature of *B. botulinus* toxin can be undertaken as soon as more is known concerning the life processes of this organism. The purpose of this investigation is to apply some of the customary nitrogen methods to the analysis of the bacterial split products in a more concentrated medium than has hitherto been employed.

Before discussing the procedures adopted in the personal investigations, it is well to review briefly some of the theories of toxin formation and a few of the chemical characteristics of *B. botulinus* observed by others. The attempts of early investigators to obtain bacterial growth on concentrated mediums also deserve careful consideration.

Novy¹ defines toxins as "specific bacterial poisons made within the cell by a synthetic process." Attempts have been made to isolate the toxin of *B. botulinus* in pure form first by Brieger and Kempner² and recently by Schübel.³ In neither investigation was it possible to separate the toxin completely from protein substances in the medium; consequently the protein nature of *B. botulinus* toxin has not been proved conclusively. Van Ermengem,⁴ in his early studies, concluded that the botulinus toxin is an exotoxin, while Dozier⁵ advanced experimental evidence, which indicates that the bacterial cell is the matrix of the poison.

Future experiments may demonstrate that protein is unnecessary for the production of the toxin of *B. botulinus*. On the other hand, it has been definitely shown that *B. botulinus* utilizes protein material. To determine whether proteolysis bears a direct relation to toxin production or to other phases of its life cycle, requires further intensive investigation. Numerous publications bear out the conclusion that

Received for publication, Nov. 9, 1924.

¹ Laboratory Work in Bacteriology, 1899, p. 84.

² Deutsch. med. Wchnschr., 1897, 33, p. 521.

³ Arch. f. exper. Path. u. Pharmakol., 1923, 96, p. 193.

⁴ Kolle und Wassermann, 1912, 4, p. 909.

⁵ Jour. Infect. Dis., 1924, 35, p. 134.

B. botulinus is a putrefactive anaerobe. Kahn⁶ classifies this species as strongly proteolytic after a study of its action on native protein and gelatin. Heller⁷ also observed proteolysis in a meat medium. The breakdown of peptone by *B. botulinus* has been demonstrated by De Bord.⁸ Reddish and Rettger⁹ object to gelatin liquefaction and peptone breakdown as criteria of proteolysis, since these substances are not native proteins. Rettger, Berman and Sturges¹⁰ have demonstrated that amino-acid-free peptone cannot generally be anabolized by bacteria. Bainbridge¹¹ has also shown that most bacteria are incapable of utilizing proteins if their split products are totally absent.

A number of investigators have attempted to measure proteolytic activity by nitrogen metabolism studies. Kendall, Day and Walker¹² were unable to demonstrate significant nitrogenous changes in *B. botulinus* cultures. De Bord⁸ studied the amino and ammonia nitrogen changes in 2% Difco peptone with and without glucose. He concluded that amino acid production can merely serve as an approximate index of proteolysis. Wagner, Dozier and Meyer¹³ measured proteolysis in terms of nonprotein nitrogen increase or protein decrease. This fraction more nearly represents the true proteolytic action of an anaerobe.

The effect of concentrated mediums on bacterial growth was first studied by Kappes.¹⁴ The water content rather than any specific chemical composition was investigated. A concentration of 25% solid material was found unfavorable for growth of certain aerobes. Later, Wolf¹⁵ and Jorns¹⁶ were able to obtain growth of *B. pyocyaneus* and *B. prodigiosus* on a medium which contained 60% solids.

Since the investigation of Burrows and Neymann¹⁷ on the toxicity of the amino acids to chicken embryo cells, these compounds have been considered inhibitive to bacterial growth. The commercial peptones contain large quantities of amino acids, consequently higher concentrations than 2% and 4% peptone have apparently not been tried. Long¹⁸ suggests the possibility of the inhibitive effect of amino acids to

⁶ Jour. Med. Res., 1922, 43, p. 155

⁷ Jour. Bacteriol., 1922, 7, p. 1.

⁸ Ibid., 1923, 8, p. 7.

⁹ Ibid., 1924, 9, p. 13.

¹⁰ Ibid., 1916, 1, p. 15.

¹¹ Jour. Hyg., 1911, 11, p. 341.

¹² Jour. Infect. Dis., 1922, 30, p. 141.

¹³ Ibid., 1924, 34, p. 63.

¹⁴ Dissertation, Leipzig, 1890.

¹⁵ Arch. f. Hyg., 1898-99, 34, p. 200.

¹⁶ Ibid., 1907, 63, p. 123.

¹⁷ Jour. Exper. Med., 1917, 25, p. 93.

¹⁸ Am. Rev. Tuberc., 1922, 5, p. 857.

the tubercle bacillus. Meyer and Stickel¹⁹ took the findings of Burrows and Neymann into consideration when they recommended a dilution of certain culture mediums. Wyon and McLeod²⁰ have recently contributed experimental evidence that amino acids are inhibitive to the growth of certain aerobes in relatively low concentrations. The highest point of tolerance was less than 2.5% glycocoll or an amino nitrogen content of less than 0.5%. Anaerobic bacteria have not been tested in mediums with high concentrations of amino acid nitrogen.

The low concentration of nitrogen in peptone solutions, which constitute the usual culture medium, is an objectionable factor in chemical studies. Unless very large samples are analyzed, the actual amount of nitrogenous material determined is exceedingly small; consequently, the percentage error incurred is very great. Furthermore, a concentrated medium enables the bacteria to exert greater chemical activity provided other environmental factors are favorable. As a rule, the peptone content of a medium has not exceeded 5%.

The first series of my experiments dealt with attempts to secure growth in high concentrations of peptone. Five strains of *B. botulinus* produced heavy growth in 25% Difco peptone sterilized by passing it through sterile Chamberland filter candles. Strain 97 was tested in both filtered and autoclaved peptone in concentrations ranging from 25 to 40%. Growth ceases in 35% peptone having an amino nitrogen content of 1.2%, but heavy growth is still obtained on a peptone solution containing 1.06% amino nitrogen.

Comparisons of growth and toxicity of *B. botulinus* in 25% and 2% Difco peptone were made. The logarithms of the bacterial counts at 6 hour intervals, with an inoculum of detoxified spores are indicated in the chart.

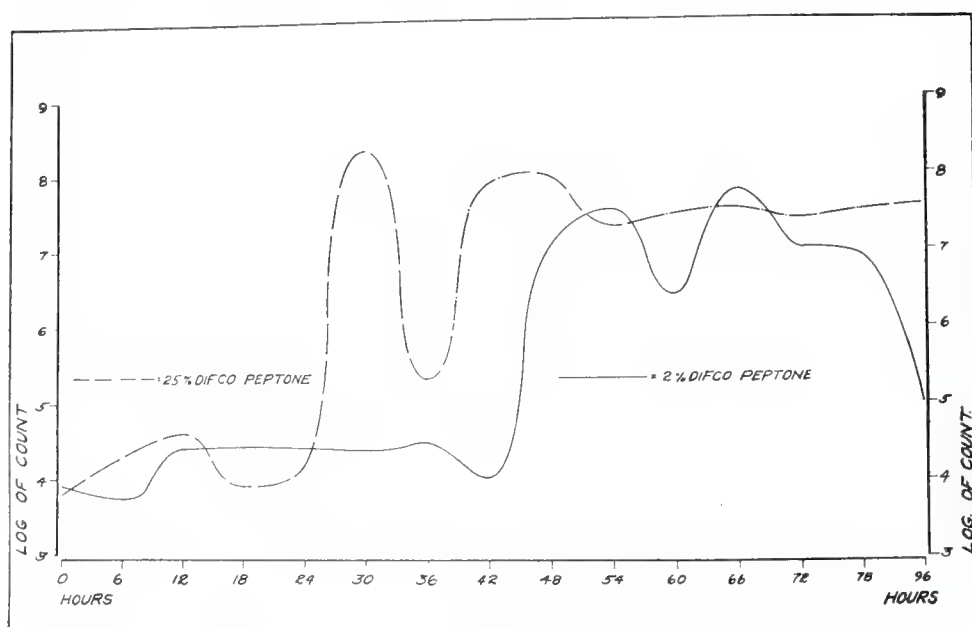
The count curves indicate that the environmental conditions of the 25% peptone are more favorable than the 2% for the germination of the spores of *B. botulinus*. Aside from more rapid reproduction, the actual numbers are greater in the 25% peptone over a period of 96 hours. The maximum count was 204,000,000 as compared to 57,500,000 in the 2% peptone. The high buffer value of the 25% peptone may possibly contribute largely to the favorable environmental conditions. If the buffer value is determined by measuring the amount of strong alkali

¹⁹ Jour. Infect. Dis., 1918, 23, p. 68.

²⁰ Jour. Hyg., 1923, 21, p. 376.

required to cause a P_H change over a measured range, according to Van Slyke,²¹ the value of the 25% peptone is 0.0625 as compared to 0.0256 of the 2% over the same range of P_H , namely, 7.4-8.5.

The toxicity was tested on white mice after one week's incubation. The M L D for mice of the toxin produced by the 25% peptone was 0.000001 c.c., while the M L D of the toxic 2% peptone was 0.001 c.c. On the other hand, when the toxin tests were made after one week's incubation after visible growth of each, the situation is different. The



A comparison of the growth of detoxified spores of *B. botulinus*, strain 97, in 25% and 2% Difco peptone.

M L D for mice of the 25% centrifuged culture is 0.000001 c.c. and of the 2% is also 0.000001 c.c.

An experiment was next executed to determine the necessity of the removal of bacterial bodies. For a time, Chamberland filtered candles were employed, but an inconsistent adsorption of nitrogenous material resulted. Centrifugalization offered another means of removal. By this method, most of the bacteria and all precipitates formed during growth were removed. By total nitrogen determinations and bacterial counts on centrifuged and uncentrifuged material, no determinable nitrogen was found in the bacterial bodies removed. The remaining

²¹ Jour. Biol. Chem., 1922, 52, p. 525.

bacteria were fewer than those removed. Consequently, it is permissible to assume that the nitrogen content of the total number of bacteria present in any sample, approximately 1,000,000,000 bacilli per 5 c.c., is negligible. The only advantage of centrifugalization previous to analysis is the removal of precipitates and the mucous substances formed during bacterial growth, which interfere with the accurate measurement of samples. These findings also indicate that little can be said of the structural composition of the bacterial bodies as they occur in suspension in a rich culture medium. Structural composition may be better determined by analysis of bacterial bodies separated from the medium in which they are grown. The composition of the bacterial bodies of *B. botulinus* will be studied at a later date by analysis of washed and dried organisms.

COMPARISON OF AMMONIA AND AMINO NITROGEN METHODS

Several methods are known for the determination of ammonia nitrogen and amino nitrogen. An attempt was made to test the applicability of some of these procedures for the analysis of Difco peptone solutions. Colorimetric methods for ammonia were not considered, because the amount of ammonia present in the cultures was sufficient to make titrimetric methods more accurate. The aeration method of Folin²² is probably one of the least laborious and most satisfactory procedures. This method has been modified repeatedly by Folin and by other workers. The chief modification has been in the choice of the alkali for the liberation of ammonia. A number of these alkalis were tested for their efficiency for complete liberation of ammonia from an ammonium sulphate solution and for their hydrolytic effect on Difco peptone. In the first test, different concentrations of ammonium sulphate were aerated with concentrated sodium hydroxide, saturated sodium chloride and sodium hydroxide, anhydrous sodium carbonate, calcium hydrate and Folin's mixture of 10% sodium carbonate and 15% potassium oxalate. The aerating system employed was an arrangement recommended by Folin and his associates,²³ according to illustrations in standard textbooks. The results obtained are tabulated in Table 1.

The 45% sodium hydroxide is the only alkali in the group that will permit the quantitative removal of ammonia from an ammonium sulphate

²² Ztschr. f. physiol. Chem., 1902, 37, p. 161.

²³ Jour. Biol. Chem., 1912, 11, p. 493.

solution during 2½ hours of rapid aeration with this equipment. Even concentrated sodium hydroxide is not sufficient if the volume in the liberating tube is increased to 25 c.c. The hydrolytic effect of the alkalis referred to above and more dilute solutions of sodium hydroxide on Difco peptone was tested as indicated in table 2.

Folin's mixture, sodium carbonate, calcium hydrate, and 1% sodium hydroxide do not hydrolize 25% Difco peptone. Another attempt was made to remove the ammonia quantitatively from an ammonium sulphate solution by aeration with these 4 alkalis with the

TABLE 1
TEST OF ALKALIS FOR AMMONIA LIBERATION WHEN ORDINARY OPEN GLASS TUBE IS USED FOR CONDUCTING AIR INTO THE AMMONIA MIXTURE

Material Aerated *	Calculated Ammonia Nitrogen Present, Mg.	% Ammonia Nitrogen Recovered
5 c.c. (NH ₄) ₂ SO ₄ soln. + 10 c.c. NaOH (conc.).....	1.994	100.0
5 c.c. (NH ₄) ₂ SO ₄ soln. + 10 c.c. NaOH (conc.) + 10 c.c. sat. NaCl..	1.994	97.6
5 c.c. (NH ₄) ₂ SO ₄ soln. + 3 gr. Na ₂ CO ₃ + 10 c.c. dist. H ₂ O.....	1.994	21.0
5 c.c. (NH ₄) ₂ SO ₄ soln. + 3 gr. Ca(OH) ₂ + 10 c.c. dist. H ₂ O.....	1.994	57.4
5 c.c. (NH ₄) ₂ SO ₄ soln. + 10 c.c. (10% Na ₂ CO ₃ + 15% K ₂ C ₂ O ₄).....	1.994	67.8
5 c.c. (NH ₄) ₂ SO ₄ soln. + 10 c.c. NaOH (conc.).....	8.542	99.7
5 c.c. (NH ₄) ₂ SO ₄ soln. + 10 c.c. sat. NaCl + NaOH (conc.).....	8.542	94.5
5 c.c. (NH ₄) ₂ SO ₄ soln. + 3 gr. Na ₂ CO ₃ + 10 c.c. dist. H ₂ O.....	8.542	75.4
5 c.c. (NH ₄) ₂ SO ₄ soln. + 3 gr. Ca(OH) ₂ + 10 c.c. dist. H ₂ O.....	8.542	90.5
5 c.c. (NH ₄) ₂ SO ₄ soln. + 10 c.c. (10% Na ₂ CO ₃ + 15% K ₂ C ₂ O ₄).....	8.542	94.2
5 c.c. (NH ₄) ₂ SO ₄ soln. + 10 c.c. NaOH (conc.) + 10 c.c. dist. H ₂ O.,	22	97.8
5 c.c. (NH ₄) ₂ SO ₄ soln. + 10 c.c. NaOH + 10 c.c. NaCl.....	22	98.1
5 c.c. (NH ₄) ₂ SO ₄ soln. + 3 gm. Na ₂ CO ₃ + 20 c.c. dist. H ₂ O.....	22	70.2
5 c.c. (NH ₄) ₂ SO ₄ soln. + 3 gm. Ca(OH) ₂ + 20 c.c. dist. H ₂ O.....	22	63.4
5 c.c. (NH ₄) ₂ SO ₄ soln. + 10 c.c. (10% Na ₂ CO ₃ + 15% K ₂ C ₂ O ₄) + 10 c.c. dist. H ₂ O	22	62.8

* Controls contained no ammonia.

aid of improved equipment. A glass tube ending in a perforated bulb was employed in the liberating tube as well as in the receiving tube, and special care was taken that the bulb nearly touched the bottom of the large test tube. With these precautions, a better distribution of air throughout the mixture was obtained. This is the procedure recommended by Van Slyke and Cullen.²⁴ The findings are stated in table 3.

This experiment indicates that of the 5 alkalis and alkali mixtures, 1% sodium hydroxide and sodium carbonate only may be used with safety for the removal of ammonia from 25% Difco peptone. Ammonia as well as amino nitrogen is formed during hydrolysis; consequently, a stronger alkali would produce a positive error in the ammonia determination.

²⁴ Jour. Biol. Chem., 1914, 19, p. 211

By either Folin's²⁵ colorimetric or Van Slyke's²⁶ gasometric method, the removal of ammonia is necessary before accurate determination of amino nitrogen is made. Permutit is recommended by Folin for the previous removal of ammonia. This reagent was tested for its suitability by determining the percentage of adsorption of a pure amino acid. A similar test was made with a 25% Difco peptone solution. Eight and seven-tenths % of the amino nitrogen was found to be adsorbed from a glycocoll solution after shaking with permutit, while as much as 15% of the amino nitrogen of Difco peptone is adsorbed. Therefore it was necessary to employ some other means of removing

TABLE 2
HYDROLYTIC EFFECT OF VARIOUS ALKALIS ON 25% DIFCO PEPTONE IN THE COLD

Material Aerated	Amino Nitrogen per 100 C c. before Addition of Alkali,* Mg.	Amino Nitrogen per 100 C c. after 2½ Hrs. Aeration with Alkali, Mg.	% Increase in Amino Nitrogen
5 c c. peptone + 10 c c. 45% NaOH.....	585.5	1588.2	64
5 c c. peptone + 10 c c. 10% NaOH.....	585.5	1040.9	44
5 c c. peptone + 10 c c. 1% NaOH.....	585.5	587.4	0†
5 c c. peptone + 10 c c. 45% NaOH and 10 c c. sat. NaCl solution	585.5	1534.6	62
5 c c. peptone + 10 c c. dist. H ₂ O and 3 gm. Na ₂ CO ₃	585.5	585.2	0†
5 c c. peptone + 10 c c. dist. H ₂ O and 3 gm. Ca(OH) ₂ ..	585.5	674.2	14
5 c c. peptone + 10 c c. (10% Na ₂ CO ₃ and 15% K ₂ C ₂ O ₄)..	585.5	582.1	0†

* The ammonia nitrogen content in 25% peptone was sufficiently low that the amino nitrogen could be accurately determined without the previous removal of ammonia.

† The differences are within the limits of experimental error, and may be disregarded.

ammonia previous to the amino nitrogen determination. One % sodium hydroxide with aeration was employed for this purpose.

The accuracy of Folin's and Van Slyke's amino nitrogen methods was studied, and comparisons were made by several determinations on pure glycocoll solutions and on 2% Difco peptone solutions before and after bacterial growth. Contrary to the findings of Green, Sandiford and Ross,²⁷ who compared the two methods for the determination of the amino acids in blood, Van Slyke's gasometric method gave higher results than Folin's colorimetric method. The results of such a comparison are largely dependent on the presence of certain amino acids in the mixture, since the same amino acids do not react to the same extent in the two methods. Both methods have their defects. By neither method can anything but a comparative figure be obtained in a complex

²⁵ Ibid., 1922, 51, p. 377.

²⁶ Ibid., 1912, 12, p. 275.

²⁷ Ibid., 1924, 58, p. 845.

nitrogenous mixture such as peptone solutions. Creatinin as well as ammonia is known to react appreciably with nitrous acid. Wilson²⁸ found that 16% of one nitrogen of creatinin reacted in 3 minutes. This percentage was checked by a determination on a standard creatinin solution. The creatinin in any sample analyzed was insufficient to produce a change in the reading of the gas buret.

Some difficulty was encountered in the shaking of the Van Slyke apparatus because of an overloaded alternating current to which the motor was connected. Often the speed of the shaking could not well be regulated. This condition was alleviated by employing a D. C. motor which was attached to a storage battery. The same result could have been obtained by attaching the motor to any other source of direct current. If the stopcocks of the shaking apparatus were kept well greased, no difficulties similar to those of De Bord⁸ were encountered with Van Slyke's method.

TABLE 3

TEST OF ALKALIS FOR AMMONIA LIBERATION WHEN A GLASS TUBE ENDING IN A PERFORATED BULB IS EMPLOYED FOR CONDUCTING AIR INTO THE AMMONIA MIXTURE

Material Aerated	Calculated Ammonia Nitrogen, Mg.	% Nitrogen Recovered
5 c.c. (NH ₄) ₂ SO ₄ soln. + 10 c.c. 1% NaOH.....	25.97	100
5 c.c. (NH ₄) ₂ SO ₄ soln. + 10 c.c. dist. H ₂ O and 3 gm. Na ₂ CO ₃	25.97	100
5 c.c. (NH ₄) ₂ SO ₄ soln. + 10 c.c. dist. H ₂ O and 3 gm. Ca(OH) ₂	25.97	92
5 c.c. (NH ₄) ₂ SO ₄ soln. + 10 c.c. (10% Na ₂ CO ₃ and 15% K ₂ C ₂ O ₄)...	25.97	82

Recently, Lamson²⁹ tested Van Slyke's method for its reliability in metabolism studies of bacteria. This writer states: "It is known that in any series of direct measurements, in spite of the most careful technique, the values will, in general, not be the same. Such indeterminate errors as produce the differences in the observed quantities follow the law of chance. . . . The most probable value of this quantity is given by the arithmetical mean of the series. . . . It can be shown by the theory of probability that an arithmetical mean computed from n equally probable observations is the square root of n times as reliable as any one observation." In table 2 of his publication, Lamson's maximum deviation from the mean was 6.6%, his maximum departure was 12%. In table 3, the maximum deviation from the mean was 9.5% while his maximum departure was 16.6%.

²⁸ Ibid., 1923, 56, p. 183.

²⁹ Jour. Bacteriol., 1924, 9, p. 307.

In order to establish the reliability of Van Slyke's method for the determination of amino nitrogen in this particular problem, an experiment similar to those executed by Lamson, substituting 25% Difco peptone for the more dilute mixtures, is very interesting. Instead of 2 c.c. samples, 0.2 c.c. samples were analyzed. A calibrated, graduated pipet was necessarily employed for measuring. The samples were washed from the measuring tube of the Van Slyke apparatus with 1 c.c. of distilled water. The 10 consecutive results are tabulated in table 4. Unfortunately, there was a change in temperature during the course of the experiment, but temperatures were recorded at the time of each reading of the gas buret. Without applying Lamson's elimination formula for a doubtful observation, the maximum deviation from the

TABLE 4
A SERIES OF DETERMINATIONS OF AMINO NITROGEN IN 25% DIFCO PEPTONE

Temperature,* C.	Factor	Sample	Reading	Correc- tion†	Vol. Gas	Mg. Nitrogen
19.5	0.5675	2 c.c. 25% peptone	2.29	0.15	2.14	1.21445
19.0	0.5690	2 c.c. 25% peptone	2.30	0.15	2.15	1.22335
18.5	0.5702	2 c.c. 25% peptone	2.24	0.15	2.09	1.151818
18.0	0.5715	2 c.c. 25% peptone	2.29	0.15	2.14	1.22361
18.0	0.5715	2 c.c. 25% peptone	2.23	0.15	2.08	1.18872
17.5	0.573	2 c.c. 25% peptone	2.27	0.15	2.12	1.21476
17.0	0.5745	2 c.c. 25% peptone	2.23	0.15	2.08	1.19496
17.0	0.5745	2 c.c. 25% peptone	2.23	0.15	2.08	1.19496
17.0	0.5745	2 c.c. 25% peptone	2.22	0.15	2.07	1.189215
17.0	0.5745	2 c.c. 25% peptone	2.25	0.15	2.1	1.20645

* Barometric pressure 756 mm.

† Corrections 1 c.c. H₂O + 0.01 c.c. Eastman's octyl alcohol 0.14, 0.15, 0.14, 0.14, 0.17, 0.16; mean 0.15.

mean was 4.2% and the maximum departure was 5.9%. The deviation from the mean of one doubtful observation was 4 times as great as the average deviation from the mean omitting the doubtful observation. Lamson expresses the opinion that the discarding of such an observation is permissible. The remaining 9 observations show a maximum deviation from the mean of 1.3+ % and a maximum departure of 2.8%. This accuracy is sufficient for the analysis of variable materials such as bacterial cultures. Therefore the preference of Van Slyke's method to any other now devised for appreciable amounts of amino nitrogen is justified as practiced in this laboratory.

It was possible to repeat amino nitrogen determinations on control material under different atmospheric conditions with less than 1% error, checking determinations to 0.03 c.c. each time.

The formol titration method of Henriques and Sørensen³⁰ was not tested. Wilson³¹ considers this method preferable for comparative analyses. Brown³² has indicated the precautions necessary if Sørensen's technic is employed for the analysis of bacterial cultures.

METHODS ADOPTED

The total nitrogen was determined in order to detect any loss of nitrogen either by the formation of gases or by the manipulation of the cultures before analysis. Nonprotein nitrogen determinations indicate to what extent the protein is broken down. The Gunning³³ modification of Kjeldahl's method was chosen for both total nitrogen and nonprotein nitrogen determination. The ammonia was distilled through a glass tube ending in a perforated bulb below the surface of the acid, instead of employing a condenser. By this procedure, the volume of the distillate was kept low, and thus the detection of the end point in titration was facilitated. The precipitation method of Folin and Wu³⁴ was utilized for the removal of the protein fraction previous to nonprotein nitrogen determination.

Ammonia, urea, creatinin, creatin and amino nitrogen were quantitatively estimated in order to secure data concerning the compounds which are utilized or produced as a result of bacterial growth. Folin's²² aeration method for ammonia, with 1% sodium hydroxide as the liberating agent, was chosen. The urease method of Van Slyke and Cullen²⁴ was the only applicable method for urea estimation. The xanthhydrol method of Fossé³⁵ was of no value on account of the minute amounts of urea present, if any. Creatin and creatinin nitrogen was determined by Folin's³⁶ microchemical method. Consistent results could be obtained only when creatin was hydrolyzed by autoclaving. As previously mentioned, Van Slyke's²⁶ method for amino nitrogen was selected.³⁷

Samples containing the total nitrogen and samples containing only nonprotein nitrogen were hydrolyzed 24 hours with hydrochloric acid,

³⁰ Ztschr. f. physiol. Chem., 1910, 64, p. 120.

³¹ Jour. Biol. Chem., 1923, 56, p. 191.

³² Jour. Bacteriol., 1923, 8, p. 245.

³³ Ztschr. f. anal. Chem., 1889, 28, p. 188.

³⁴ Jour. Biol. Chem., 1919, 38, p. 81.

³⁵ Ann. de l'Inst. Pasteur, 1916, 30, p. 525.

³⁶ Jour. Biol. Chem., 1914, 17, p. 469.

³⁷ The procedure employed was varied slightly from the description in the standard textbooks. Based on the diagram of Van Slyke's apparatus in Hawk's Practical Physiological Chemistry, 8th edition revised, p. 87, the procedure employed was as follows: After the displacement of air by nitric oxide by closing stopcock *c* with stopcock *a* open, and shaking, stopcock *a* was closed and *f* closed but *c* was open. The mixture was shaken out 2 minutes. Then *c* was closed and *f* opened. From this point, the procedure was the same as that described by Van Slyke. It was understood that this slight change was suggested by Dr D. D. Van Slyke during his stay in this laboratory.

and ammonia and amino nitrogen determined. The precipitate formed during hydrolysis is designated as humin nitrogen. These hydrolyses offered a means of expressing protein and peptid changes in terms of amino nitrogen.

PREPARATION OF MEDIUM AND INOCULATION

A 25% peptone, 0.5% sodium chloride solution was chosen as a suitable medium for the study of the nitrogen metabolism of *B. botulinus* utilizing the nitrogen methods mentioned above. The reaction of this concentration of Difco peptone in distilled water was P_H 7.4, consequently addition of alkali to produce a favorable reaction was unnecessary. The medium was sterilized by passing it through a sterile Chamberland filter candle into a large flask. It was well mixed, transferred to small flasks in 250 c.c. portions, covered with a layer of petrolatum and incubated to test its sterility. This method of sterilization, although slightly more time consuming than autoclaving, has the advantage of giving a clear solution. Precipitates not only interfere with accurate measurement of samples, but undoubtedly share in the nutritive value of the medium and would therefore have to be accounted for if present. Approximately 10 c.c. portions of the medium were placed in test tubes under petrolatum and incubated, to be used later as seed tubes for the flasks.

The stock cultures of *B. botulinus*, strains 97 and 38, A types, and strains 65 and 6, B types, were selected. These cultures had been purified by picking a single colony in deep agar. The procedure for inoculation was as follows: One tenth of one c.c. of a beef heart peptic digest culture, which had been incubated 24 hours, was the inoculum for the seed tube. This tube was incubated 24 hours, and 1 c.c. of its contents was then transplanted to the flasks which were later analyzed. The seed tube was tested for purity by the shake tube method. After approximately 96 hours' incubation, the contents of the culture flasks were also tested for purity by observing the appearance of the colonies formed in deep agar. Smears of the cultures were stained and examined under the microscope. Plate counts were made from each culture, following the technic of Wagner, Dozier and Meyer.¹³ The reaction of each control and culture was determined before analysis by the colorimetric method of Clark and Lubs.³⁸ Some of the bacterial bodies and insoluble material produced during growth were removed by centrifugalization before the cultures were analyzed. Evaporation during sedimentation was diminished by a layer of paraffin oil on the surface of the liquid. Toxin tests of each culture were made on white mice. The M L D of cultures 97 and 38 for mice was 0.00001 c.c., while the M L D of a culture of strain 65 was 0.1 c.c. and strain 6 was nontoxic after 4 days' growth.

EXPERIMENTAL RESULTS

The experimental findings resulting from the analysis of duplicate controls and cultures of strains 97, 65, 38 and 6 of *B. botulinus* grown in a medium consisting of 25% Difco peptone and 0.5% sodium chloride in distilled water, are given in table 5 in terms of percentage of total nitrogen.

³⁸ Jour. Bacteriol., 1917, 2, p. 1.

TABLE 5

NITROGEN CHANGES IN 25% DIFCO PEPTONE PRODUCED BY FOUR STRAINS OF *B. BOTULINUS**

Series I	Reac- tion	Viable Bacteria per C c.	Non- protein Nitro- gen, % Total Nitrogen	Am- monia Nitro- gen, % Total Nitrogen	Urea Nitro- gen, % Total Nitrogen	Creatin Nitro- gen, % Total Nitrogen	Creat- inin Nitro- gen, % Total Nitrogen	Amino Nitro- gen, % Total Nitro- gen	Hydro- lyzed Total Amino Nitro- gen, % Total Nitrogen	Hydro- lyzed Non- protein Amino Nitro- gen, % Total Nitro- gen	Hydro- lyzed Total Am- monia Nitro- gen, % Total Nitro- gen	Hydro- lyzed Non- protein Am- monia Nitro- gen, % Total Nitro- gen	Total Humin Nitrogen or Ocluded Nitrogen of Hydro- lysis, % Total Nitrogen	Non- protein Humin Nitrogen or Ocluded Nitrogen of Hydro- lysis, % Total Nitrogen	Total Nitrogen, Milli- grams per 100 C c.	Toxicity§ MLD for Mice	
Series I																	
Control 1.....	7.1	0.0	92.4	0.28	0.05	4.3	1.8	19.1	70.3	64.3	3.08	2.26	0.44	0.36	3109.6		
Control 2.....	7.1	0.0	91.9	0.27	0.05	4.3	1.7	18.9	69.8	63.8	3.6	2.24	0.0	0.33	3132.2		
Culture 1 Strain 97A 95 hours..	6.8	52.5*	94.5	11.9	0.53	3.6	1.7	20.2	62.5	61.0	19.2	19.2	3127.7	0.0001 c.c.	
Culture 2 Strain 97A 95 hours..	6.4	26.0	94.4	13.4	0.64	3.3	1.7	20.8	56.9	56.9	16.9	15.6	3110.2	0.0001 c.c.	
Culture 1 Strain 65B 95 hours..	6.3	700.0	98.8	15.2	0.17	5.2	1.4	20.4	58.4	52.9	22.7	21.5	0.92	2.50	3102.6	0.1 c.c.	
Culture 2 Strain 65B 95 hours..	6.5	530.6	97.4	14.6	0.77	7.7	1.4	20.4	56.5	56.5	20.2	20.2	1.11	2.2	3115.2	0.1 c.c.	
Series II																	
Control.....	7.4	0.0	85.4	0.31	0.0	0.11	0.47	19.1	66.4	61.2	1.7	1.4	0.36	0.38	3663.4†	0.0001 c.c.	
Culture 1 Strain 38A 94 hours..	7.0	8.0	96.1	10.5	0.0	0.43	0.10	33.9	61.0	59.0	19.3	19.3	0.74	0.81	3369.3	0.0001 c.c.	
Culture 2 Strain 38A 94½ hours	7.25	4.05	93.0	5.1	0.0	0.82	0.21	36.1	60.9	56.8	17.2	17.2	0.58	1.6	3515.6	0.0001 c.c.	
Culture 1 Strain 6B 96 hours...	7.1	156.0	92.8	13.8	0.0	0.66	0.29	16.3	58.7	54.3	17.3	17.3	0.44	0.39	3659.4		
Culture 2 Strain 6B 95 hours...	7.1	331.0	94.2	14.0	0.0	0.40	0.26	14.6	59.4	54.9	18.5	17.7	0.45	1.15	3675.3		
A																	
% change 97 I.....	2.4+†	11.62+	0.48+	0.7-	0.0	1.2+	7.6-	3.1-	15.9+	16.9+	0.36	0.38			
% change 97 II.....	2.3+	13.12+	0.59+	1.0-	0.0	1.8+	13.2-	7.2-	13.6+	13.3+	0.74	0.81			
% change 38 I.....	10.7+	10.19+	0.0	0.3+	0.37-	14.1+	5.4-	2.2-	17.6+	17.9+	0.58	1.6			
% change 38 II.....	7.6+	4.79+	0.0	0.7+	0.26-	17.0+	5.4-	4.4-	15.5+	15.8+	0.44	0.39			
B																	
% change 65 I.....	6.7+	14.92+	0.12+	0.9+	0.3-	1.4+	11.7-	11.2-	19.4+	19.2+	1.6+	2.2+			
% change 65 II.....	5.3+	14.32+	0.72+	3.4+	0.3-	1.4+	13.6-	7.6-	16.9+	17.9+	0.9+	1.9+			
% change 6 I.....	7.4+	13.49+	0.0	0.6+	0.18-	2.8-	7.7-	6.9-	15.6+	15.9+	0.08+	0.01+			
% change 6 II.....	8.8+	13.69+	0.0	0.3+	0.21-	4.5-	7.0-	6.3-	16.8+	16.3+	0.09+	0.77+			

* Strain 97, 215 million in 24 hours.

† + indicates increase; -, decrease.

‡ Medium for series II not well mixed, therefore total nitrogens do not check.

§ Controls and cultures of strain 6 were not toxic.

DISCUSSION OF RESULTS

The findings presented in table 5 indicate marked changes in the nitrogen metabolism of *B. botulinus*, which are less apparent in very dilute peptone mediums. The results of De Bord⁸ with the same organism grown in 2% Difco peptone are similar with respect to ammonia and amino nitrogen, but a 25% peptone medium affords a greater magnitude in the chemical changes. An acid reaction results from the growth of both the A type and B type strains. Hydrogen-ion concentration and ammonia production run consistently parallel. The excess of volatile and fixed acids is apparently sufficient to overcome the buffer action of the peptone as well as the ammonia production; consequently, an acid reaction results. Jacoby³⁹ advanced the hypothesis that the soluble catabolic products of the normal cell are eliminated in the form of ammonia. The large amount of ammonia formed by *B. botulinus* suggests that it is undoubtedly an end product of its metabolism. The control medium is practically ammonia free. After 96 hours of bacterial growth, approximately $\frac{1}{6}$ of the total nitrogen has been converted to ammonia nitrogen. The increase in urea nitrogen is insignificant. The apparent nitrogen is probably ammonia nitrogen formed by enzymic action of the cultures while being warmed in the incubator. This was tested in a number of cases and found to be true.

Creatinin remains practically constant, with a slight decrease as a result of the growth of the B type strain. Creatin is decreased by the A type strain and increased considerably by the B type strain.

The maximum percentage error of the total nitrogen determined in the controls and the cultures in series 1 is less than 1%. Consequently, it may be assumed that no determinable nitrogen is lost in the form of gases or in the bacterial bodies and the precipitates removed by centrifugalization. The deviation of the total nitrogen of the control in series 2 and the total nitrogen of strains 38 and 6 may be explained by the fact that the culture medium was not mixed before division into smaller portions.

Little can be said of the amino nitrogen fraction except that the slight increase indicates a rejection of some amino acids by the organism as food. Deaminization of the amino acids by *B. botulinus* is apparently a selective process. The decrease in the protein and peptid nitrogen is approximately equal to the ammonia nitrogen formed. Thus it would

³⁹ Ztschr. f. physiol. Chem., 1900, 30, p. 149.

seem that ammonia production is almost entirely a result of deamination. If the protein fraction of peptone may be considered a true protein, its decrease is a measure of the proteolytic activity of *B. botulinus*, and the decrease of peptid nitrogen a measure of peptolysis if the peptid disintegration may be designated as such. The ammonia increase during hydrolysis of proteins and peptones is sometimes considered to be roughly parallel to glutamic acid, but the ammonia increase during hydrolysis of peptone is insignificant until more is known of the amino acids present.

The nonamino nitrogen not determined probably consists largely of the imino nitrogen of the amino acids. There is a slight decrease in this fraction as a result of bacterial growth, as might be expected.

The loss of viability of the bacteria as growth ensues in 25% Difco peptone is much more marked by the A type than by the B type. The viability is probably decreased as a result of enzymic digestion, as indicated by Sturges and Rettger⁴⁰ and C. C. Dozier.⁵

SUMMARY AND CONCLUSIONS

As a method of sterilizing mediums to be analyzed chemically, filtration is superior to autoclaving.

Advantages of concentrated mediums for quantitative studies have been pointed out, and the inhibitive effect of high concentrations of Difco peptone to *B. botulinus* has been tested. A 30% solution of the peptone was the highest concentration producing heavy growth.

The environmental advantages of 25% Difco peptone over the 2% for the germination of *B. botulinus* spores have been demonstrated. The highest number of organisms counted in the 25% peptone was 204,000,000 as compared to 57,500,000 in the 2% peptone.

Toxin formation in 2% and 25% Difco peptone is equal provided both cultures are incubated the same length of time after visible growth appears.

Centrifugalization is preferable to filtration for the removal of bacteria from cultures to be analyzed.

Ammonia and amino nitrogen methods have been investigated experimentally, and Van Slyke's amino nitrogen method has been found valuable for the determinations in culture mediums.

The results dealing with a study of the nitrogen metabolism of 4 strains of *B. botulinus* grown in a 25% Difco peptone and 0.5%

⁴⁰ Jour. Bacteriol., 1922, 7, p. 551.

sodium chloride solution have been discussed. The most evident changes are the following: A decided decrease in the protein and peptid nitrogen fractions and a tremendous increase in ammonia nitrogen. The ammonia production is practically equivalent to the protein and peptid disintegration, which indicates that deaminization of the amino acids is responsible for most of the ammonia formed.

The decrease in the protein and peptid nitrogen is a measure of the proteolytic and peptolytic activity of *B. botulinus*.

STUDIES ON THE THERMAL DEATH TIME OF SPORES OF CLOSTRIDIUM BOTULINUM

IV. THE RESISTANCE OF SPORES TO HEAT AND THE DORMANCY OR DELAYED GERMINATION OF SPORES WHICH HAVE BEEN SUBJECTED TO HEAT *

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In previous reports¹ concerning the effect of heat on the spores of *Cl. botulinum*, we have shown that there is marked variation in the resistance of spores of the same and of different strains to heat, and that when heated spores are incubated in mediums which are suitable for their growth, there is marked variation in the times in which the surviving spores may germinate and produce vegetative growth. Our earliest experiments² suggested that the spore resistance and the delay in germination were directly proportional to the amount of heat to which the spores had been subjected, and similar observations which have been recorded by other investigators³ have been interpreted in this manner. Our later experiments with larger series of tube preparations have shown that the heat resistance of spores cannot be wholly explained in this way, and experiments which have been recorded by one of us⁴ show that there is a factor of normal dormancy in botulinus spores which must be taken into account in interpreting the delayed germination of heated spores.

It is the purpose of this report to state some of the observations which were made by a study of approximately 37,000 sealed tube

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¹ Dickson, E. C.; Burke, G. S., and Ward, E. S.: Botulism: Arch. Int. Med., 1919, 24, p. 581. Burke, G. S.: Jour. Am. Med. Assn., 1919, 72, p. 88. Dickson, E. C.; Burke, G. S.; Beck, D.; Johnston, J., and King: Ibid., 1922, 79, p. 1239.

² Dickson, Burke and Ward: Arch. Int. Med., 1919, 24, p. 581. Burke, G. S.: Jour. Am. Med. Assn., 1919, 72, p. 88.

³ Esty, J. R., and Meyer, K. F.: Jour. Infect. Dis., 1922, 31, p. 650. Estey, J. R.: Amer. Jour. Pub. Health, 1923, 13, p. 108. Estey, J. R., and Williams, C. C.: Jour. Infect. Dis., 1924, 34, p. 516. Weiss, H.: Jour. Infect. Dis., 1921, 28, p. 70.

⁴ Burke, G. S.: Ibid., 1923, 33, p. 274.

preparations⁵ in which the spores of *Cl. botulinum* were heated and observed over periods of from 28 to 39 months. As was stated in our preliminary report, the 11 strains were selected from 41 strains in a series of preliminary tests by the open tube method of Bigelow and Esty.⁶ The majority of the final tests in sealed tubes were made by heating and incubating the spores in 1% glucose peptic digest broth (P_H 7.0-7.4), which was covered by a thin layer of mineral oil, although smaller series were run in glucose broth without oil, glucose agar and brain medium; 29,421 tube preparations were run in oil-stratified broth, 4,636 in broth without oil, 1,422 in agar and 1,426 in brain medium. Approximately 50,000,000 spores were heated in each tube.

In order that the interpretation of the results of the experiments might be as uncomplicated as possible and that there might be no doubt as to the possibility of bacterial contamination of tubes in which unexpected growth might appear, the greatest precautions were taken to control every step in the experiments. The medium in which the spores were to be heated and incubated was tested for sterility by from 4 to 6 weeks' incubation at 37 C. before it was inoculated. The number of spores in the spore mixture was carefully determined for each experiment by actual count in a counting chamber. The tubes were sealed in as gas-oxygen flame within a few minutes after they were inoculated, and any tubes which cracked at any stage of the experiment were promptly discarded.

It was obviously impossible to make culture and toxin tests of the contents of all the tubes which showed signs of growth, but in every instance (except in agar preparations) in which maximum survival times or germination times exceeding 15 months are recorded, the identity of the bacterial growth within the tube was tested by subcultures in broth and in agar, and the presence of toxin was demonstrated by the inoculation of guinea-pigs. There were several instances in which tube preparations which showed signs of turbidity suggesting bacterial growth indicated survival times considerably in excess of those which are recorded, but although the medium contained some toxic substance which caused the death of the guinea-pigs within 3 or 4 days after they received injections, it was impossible to obtain positive subcultures to establish the presence of living bacteria. None of these is included in the reports which follow.

⁵ Dickson, E. C., and Burke, G. S.: *Proceed. Soc. Exper. Biol. & Med.*, 1921, 19, p. 99.

⁶ Bigelow, W. D., and Esty, J. R.: *Jour. Infect. Dis.*, 1920, 27, p. 602.

Heating Time Min.	A	B	C	D	E	F	G	H	I
95	(38) 5	(3) 5	(50) 5	(13) 5	(20) 5	(13) 5	(80) 4	(45) 4	(23) 5
100	(2) 6	(26) 8	(19) 6	(19) 6	(31) 8		(15) 5	(7) 4	(10) 6
105	(22) 8	(16) 8	(20) 6	(3) 10	(6) 7	(2) 4	(26) 5		(47) 5
110	(11) 8	(9) 11	(2) 18	(3) 10	(8) 7	(13) 6	(5) 6	(24) 6	(1) 20
115	(4) 8		(2) 11	(1) 89	(1) 8	(25) 6	(22) 7	(2) 7	(1) 20
120	(1) 18	(4) 18			(1) 20	(2) 8	(6) 7	(12) 4	
125	(1) 81	(1) 20	(3) 89	(1) 89		(13) 7	(8) 8	(7) 4	(1) 20
130	(1) 89	(1) 81		(1) 89	(1) 20	(1) 17		(2) 17	
135			(1) 138	(1) 20			(1) 10	(1) 10	
140		(1) 89		(1) 89				(1) 107	
145					(1) 201	(1) 26	(1) 19	(1) 10	
150						(1) 17		(1) 81	(1) 81
155						(1) 114			
160				(1) 107					
165								(1) 325	
170			(1) 119						

Chart 1.—Irregular distribution of the positive tubes (shaded squares) with the occurrence of skips in heating test of botulinus spores (strain 58) in agar at 100 C. Each square represents one tube, and 9 duplicate tubes were removed at each tube removal time. The numbers within the circles indicate the number of colonies which developed in each tube and the numbers within the oblong spaces indicate the period of dormancy in days. Each blank square indicates a tube which has remained sterile for 33 months. Date of heating, Feb. 10, 1922.

THE OCCURRENCE OF SKIPS

One of the first observations which was made while we were still using the open tube technic of Bigelow and Esty,⁶ in fact it was the observation which made it necessary to devise the sealed tube technic⁵ and to adopt the duplicate series technic in each heating experiment, was that the survival of spores is not entirely dependent on the length of time for which they have been exposed to heat. In many instances it was found that in a single tube series set of open tubes bacterial growth would be observed in tubes which had been submitted to much longer exposure to heat than had others which remained sterile. It was only by substituting the sealed tube technic⁵ that we were able to exclude all possibility that these positive cultures were the result of contamination after the spores had been heated, and by adopting the duplicate series of tube preparations in each experiment to obtain accurate information as to the actual incidence of these skips.

The appearance of the charts of experiments in which skips have occurred is shown in charts 1 and 2. The skips have been demonstrated in all the mediums which were used and at all the temperatures at which the spores were heated, but they are more constant when the spores have been heated at the higher temperatures and when they are heated in oil-stratified broth.

It is an interesting fact that although there is no line of demarcation in the tube preparation series on one side of which all the tubes show bacterial growth from surviving spores and on the other side of which all the tubes remain sterile, there is a point in every series above which all or practically all of the tubes show early and vigorous growth and below which the occurrence of skips begins and delayed germination is observed. This point is more clearly defined when spores are heated at lower temperatures but can be recognized at the 3rd and 4th minute intervals in chart 2. It was much more clearly shown in the earlier portion of the record from which chart 1 was prepared, although it is not included in the chart. In the first 90 minutes of this heating test 9 duplicate tubes were removed at each interval of 5 minutes as in the latter half of the experiment which is charted. Every one of these 162 tubes showed bacterial growth within 5 days after they were heated, and in these which were removed from the bath within the first 60 minutes the number of colonies which appeared was too great to be counted. Beyond that point, the number of colonies in each tube became

Heating									
Time									
Min.	A	B	C	D	E	F	G	H	
0	1	1	1	1	1	1	1	1	
1	1	1	1	1	1	1	1	2	
2	2	2	2	2	4	4	4	76	
3	4	4	2	4	4	34	87		
4		4	4	4	6		117	82	
5	39		17	61				61	
6				87		76	87	87	
7	55		132		19	117	187	4	
8	87	87	87		87		117	87	
9					87			87	
10	105		87			96	87		
11	187	61							
12		76	87		87	96		153	
13				111			96	146	
14	87					105			
15				187	187				
16						82			
17		251			87		105		
18		19 mo.							
19				87		87			
20			117						
21					21 mo.				
22			96						
23							71		
24									
25									
26									
27									
28		11 mo.							

Chart 2.—Irregular distribution of the positive tubes (shaded squares) with the occurrence of skips when botulinus spores (strain 58) were heated in oil stratified broth at 115 C. The numbers within the shaded squares indicate the dormancy in days, except when months are noted. Each blank square represents a tube which remained sterile for 34 months. There were 8 duplicate series. Date of heating, Dec. 22, 1921.

rapidly less numerous and the number of skips more frequent until from the 95 minute interval on, as shown in chart 1, the number of bacteria was relatively small, despite the fact that 50,000,000 spores had been heated in each tube, and there were many skips. The inference

TABLE 1

TIME OF GERMINATION IN MONTHS AFTER BOTULINUS SPORES WERE HEATED IN OIL STRATIFIED BROTH IN SEALED TUBES

Months after Heating	Temperatures at Which Spores Were Heated. Degrees Centigrade					Total
	100	107	115	118	121	
1.....	1532	521	1039	52	432	3576
2.....	68	130	312	5	57	572
3.....	160	124	310	8	49	651
4.....	126	53	220	42	441
5.....	48	33	121	1	25	228
6.....	35	11	49	1	18	114
7.....	19	16	47	15	97
8.....	3	11	37	4	55
9.....	17	9	14	5	45
10.....	1	15	19	11	46
11.....	5	2	16	23
12.....	3	1	6	1	11
13.....	1	3	5	1	10
14.....	1	1	2	4
15.....	1	1
16.....
*	35	12	56	24	127
17.....
18.....	2	2
19.....
20.....	1	1	1	3
21.....	2	2	1	5
22.....	5	1	2	2	10
23.....	4	4	8	2	18
24.....	7	1	11	1	4	24
25.....	4	9	13
26.....	1	1	8	4	14
27.....	4	3	4	1	12
28.....	2	2	4	1	9
29.....	3	1	2	1	7
30.....	1	2	1	4
31.....	3	3	6
32.....	2	1	3
33.....	1	1	2
34.....	1	3	4
35.....	1	1	1	3
36.....
37.....	1	1
Total tubes positive.....	2092	964	2313	70	702	6141
Total tubes heated.....	3802	2905	14270	490	7954	29421

* Indicates an interval of about 5 months during which the tubes were not inspected.

is that there is an interval in the heating time during which the great majority of the spores are destroyed, but beyond which a relatively very small number of spores survive and retain ability to germinate and originate a new vegetative stage.

DORMANCY

The second interesting fact which has been demonstrated in these experiments is that the germination time of surviving botulinus spores may be greatly delayed even when they are placed in environments which are suitable for their growth and in which many of the spores germinate promptly. The results of our observations are shown in tables 1 and 2, but there is no reason to believe that they represent the maximum dormancies which may occur since every monthly inspection of the heated tubes has revealed new series of positive tubes.

TABLE 2
TIME OF GERMINATION IN MONTHS AFTER BOTULINUS SPORES WERE HEATED IN BROTH WITHOUT OIL AND IN AGAR, RESPECTIVELY, IN SEALED TUBES

Months after Heating	Plain Broth Temperature at Which Spores Were Heated					Deep Agar Temperature at Which Spores Were Heated				
	100	107	117	121	Total	100	107	115	121	Total
1.....	205	62	205	83	555	274	4	33	27	338
2.....	49	30	19	6	104	2	5	1	1	9
3.....	27	22	11	3	63	14	1	1	...	16
4.....	6	4	6	...	16	7	5	1	...	13
5.....	3	2	1	1	7	1	1
6.....	2	3	2	...	7
7.....	1	1
8.....	1	1
9.....	1	...	1
10.....
11.....	1	1
12.....
*.....	1	1
17.....
18.....	2	...	5	...	7
19.....
20.....	2	2
21.....	2	2
22.....	3	...	3
23.....
24.....
Total tubes positive.....	300	123	253	93	769	300	15	36	28	379
Total tubes heated.....	4638	392	2220	910	4638	510	96	520	300	1426

* Indicates a period of about 5 months during which the tubes were not inspected.

The greatest dormancy has been observed in those tube preparations in which the spores were heated and incubated in oil-stratified broth, the next longest in broth without oil and the shortest in agar. Because of the difficulty of recognizing the beginning of growth in brain medium that series of tube preparations has not been included in the investigation of delayed germination. From the evidence shown in chart 1 and from other agar series, there is indication that in many instances the delayed growth is produced by the germination of a single spore.

TABLE 3

MAXIMUM SURVIVAL TIME, THERMAL DEATH TIME, HEATING LAG TIME AND GERMINATION TIME OF SURVIVORS WHEN BOTULINUS SPORES ARE HEATED IN SEALED TUBES IN DIFFERENT MEDIUMS AND AT DIFFERENT TEMPERATURES

Medium	Strain	Type	100 C.				107 C.				115 C.				118 C.				121 C.			
			Survival Time	Death Time	Lag	Germination Time	Survival Time	Death Time	Lag	Germination Time	Survival Time	Death Time	Lag	Germination Time	Survival Time	Death Time	Lag	Germination Time	Survival Time	Death Time	Lag	Germination Time
Oil Stratified Broth	3	A	210	225	7	203 da	30	35	3	210 da	24	25	4	201 da	7	8	3	167 da	7	8	5	103 da
	10	B	285	300	3	23 mo	45	50	4	139 da	35	36	3	133 da	22	23	2½	140 da
	19	A	195	210	4	141 da	30	35	4	23 mo	28	29	4	142 da	15	16	3	143 da
	24	A	60	75	3	144 da	15	20	3	145 da	36	37	3	146 da	6	7	3	19 da
	31	B	7	8	3	237 da	5	6	3	..	4	5	6	81 da
	58	A	270	285	5	47 da	55	60	4	148 da	42	43	3	82 da	14	15	3	30 mo	22	23	4	188 da
	62	A	360	375	4	149 da	45	50	5	150 da	30	31	5	151 da	11	12	3	126 da	18	19	3	28 mo
	65	B	36	37	3	20 mo
	87	A	180	195	3	88 da	80	85	4	125 da	37	38	3	24 mo	25	26	3	..	14	15	3	222 da
	88	B	120	135	3	27 da	30	35	3	148 da	30	31	2½	304 da
	90	A	26	27	3	46 da
Plain Broth	10	B	225	240	3	8 da	30	35	4	21 mo	6	7	3	152 da	3	4	3	113 da
	58	A	240	255	3	60 da	40	45	3	137 da	7	8	3	213 da	4	5	3	63 da
	87	A	165	180	3	18 mo	25	30	3	72 da	5	6	3	73 da	2	3	3	72 da
Agar.....	10	B	210	225	3	42 da	20	25	4	106 da	5	6	3	95 da	3	4	3	13 da
	58	A	170	185	3	111 da	15	20	2	96 da	4	5	3	19 da	3	4	3	60 da
Brain....	10	B	270	285	4	..	40	45	3	..	8	9	3	5	6	3	..
	58	A	240	255	4	..	50	55	3	..	10	11	4	7	8	4	..

Numbers indicate minutes unless otherwise noted; da, indicates days; mo, indicates months.

MAXIMUM SURVIVAL TIME

The maximum survival time and the thermal death time of botulinus spores are shown in table 1. The thermal death time in each instance is given as the tube removal time next beyond that at which the maximum survival of spores was demonstrated, the intervals between tube removal times being 15 minutes in the 100 degree tests, 5 minutes in the 107 degree tests and 1 minute each in the 115, 118 and 121 degree tests. The survival times are stated in minutes which indicate the length of time the tubes were immersed in the heating bath, although the net time for which the contents of the tubes were held at the full temperature of the experiment is obtained by subtracting the "lag" time from the stated survival time. It is, however, doubtful whether it is preferable to state the heating time in terms of the net time of exposure to maximum heat since during the lag the temperature to which the spore is exposed is constantly approaching the desired maximum temperature of the experiment.

Charts 1 and 2 have shown that there is considerable variation in the heat resistance of spores in a given culture of a single strain of *Cl. botulinum* when the spores are heated and incubated in multiple tube series in which each tube is exposed to identical conditions. Table 3 shows clearly that there is marked variation in the heat resistance of spores of different strains of *Cl. botulinum* when exposed to similar conditions as well as in those of the same strains when heated and incubated in different mediums. In general, the resistance of the type A strains which were tested averages somewhat higher than that of the type B strains of our series, but in oil stratified broth, the maximum survival time of a type B strain (no. 10) when heated at 121 C. equals that obtained in any of the type A strains (no. 58) at that temperature, and the maximum survival times of strain 10 (type B) are consistently higher at all the test temperatures than those of strains 3, 19 and 86 which are type A strains. It is apparent from these observations that many more tests must be made before one can assume that type B strains of *Cl. botulinum* are consistently less resistant to heat than type A strains.

DISCUSSION

Repeated inspections of the heated tube preparations of botulinus spores over a period ranging from 28 to 39 months after they were heated have not necessitated any material alteration in the conclusions which were recorded in our preliminary report.⁷ The incidence of skips

⁷ Dickson; Burke; Beck; Johnston and King: Jour. Am. Med. Assn., 1922, 79, p. 1239.

has been established; the evidence that the majority of the spores are destroyed relatively quickly by relatively low degrees of heat remains unchanged, and it appears to be proved that a very small number of spores in a spore mixture may be so highly resistant to heat that they will survive prolonged exposure to degrees of heat which promptly destroy the great majority of the spores.

The only changes from the maximum survival times as reported in the preliminary report are an increase of 45 minutes for spores heated in oil stratified broth at 100 C., an increase of 5 minutes for spores heated in brain medium at 107 C., an increase of 1 minute for spores heated in brain medium at 121 C., and an increase of 15 minutes for spores heated in broth without oil at 100 C.

The most striking, and, in our opinion, the most important observation which was made in these experiments is that when botulinus spores are heated and incubated in broth which is covered with a thin layer of oil, the heat resistance time is very much greater than when they are heated in similar broth without a covering of oil. A glance at table 3 will recall that the maximum survival times of spores in oil stratified broth are considerably greater than are observed in the other mediums, and in strains 10, 58 and 87, where there are comparative readings in oil stratified broth and in plain broth at all the best temperatures, the maximum survival times are uniformly greater in the oil stratified broth. This increased heat resistance is much more apparent when the spores are heated at the higher temperatures, 115 and 121 C., which require relatively short periods of exposure to destroy them, than when they are heated at 100 C. which they survive for comparatively much longer periods.

There are at least two factors which may play a part in the causation of these results, although we are not convinced that they explain all the facts.

It can be easily demonstrated that the conduction of heat from oil to objects which are immersed in it is considerably slower than is the case when similar objects are immersed in water of like temperatures. This factor does not assume importance in the transferring of heat from the oil of the heating bath to the spore mixtures within the tubes, because in every heating experiment the actual lag was checked by temperature readings within the tubes and was essentially the same for all the tubes which were heated in each test. It may, however, play a part in determining the relative duration of exposure of spores which are

suspended in oil to the maximum heat of the experiment as compared with spores which are suspended in the broth. If it requires a longer time to bring the spores in oil to the maximum temperature of the experiment than is required for the spores in broth, the obvious result is that the spores which are immersed in oil are exposed to the maximum heat of the experiment for a shorter time than would those which are suspended in the broth, even though the temperature registration of the broth and oil may be identical and one can well imagine that when the total time of exposure to heat is short, as when the spores are heated at the higher temperatures, this secondary lag would be of much greater significance than when the heating time is more prolonged. It does not seem to be improbable that in this way one can at least partially explain the facts that there are increased resistance times in spores which are suspended in oil as compared with those which are heated in broth, that the greatest differences are noted when the spores are heated for relatively short periods at higher temperatures—115 and 121 degrees—and that the differences are relatively small when the spores are heated at lower temperatures for longer periods.

Another factor which may play a part is that spores exposed to heat in oil are exposed to conditions which, at least in degree, resemble those which obtain in dry heat sterilization. Despite the fact that there must be water vapor from the broth which lies below the oil, it is improbable that the spores which are suspended in the oil can receive the full action of the heat-moisture combination to which the spores which are suspended in the broth are subjected. In other words, it may well be that the spores which are suspended in the oil do not receive the same degree of sterilizing effect as is produced by equal degrees of heat in the presence of moisture, and that in addition to the shorter exposure to maximum heat because of the secondary lag of the oil, the spores within the oil are also exposed to less efficient sterilizing processes.

Any conclusion as to what factors may determine the delayed germination of heated spores must be postponed until the final results of the tests become available. It has been noted that when the tube preparations containing the spore mixture in oil stratified broth are frequently agitated there is an apparent increase in the numbers of tubes which show positive growth, suggesting that spores may have been dislodged from the oil and settled into the broth below where they could germinate. There is also some evidence that when the spores are suddenly exposed to changes in temperature, as when they are changed from incubator to room temperature, or vice versa, there is an increase in the

number of tubes which show growth. A similar effect of changing temperature has been noted in the germination of certain seeds,⁸ and has been mentioned by one of us (Burke) as a possible factor in connection with the delayed germination of normal unheated botulinus spores.⁴

The maximum dormancies which have thus far been recorded in our series are 37 months for spores which were heated and incubated in oil stratified broth, 22 months for spores which were heated and incubated in broth without oil and 11 months for spores which were heated and incubated in agar. It is impossible to predict whether longer dormancies may be observed in the series, but until the maximum is reached it would be premature to attempt to draw any final conclusion as to whether the normal primary dormancy of botulinus spores may fully explain the prolonged delay in germination of heated spores, or whether it may be necessary to assume that the delay is due to a secondary dormancy which is produced by the effect of the heat on the outer covering of the spore.

Whether or not the explanations which we have suggested may be correct, the fact remains that when botulinus spores are heated and incubated in broth medium which is covered with a thin layer of mineral oil, the resistance of the spores to heat and the length of time which may elapse before the spores may germinate are greatly increased. This must be of direct interest to all persons who have to do with the preservation, especially the canning, of foods, because if it is shown that thin layers of vegetable or animal oils may lead to similar results, it will be necessary to consider carefully the processing time of all foods in which these oils may exist or from which they may be liberated, and to exercise great caution in the immediate interpretation of tests to determine whether a given process is sufficient to ensure the destruction of botulinus spores.

SUMMARY

This report deals with a series of observations on the thermal death time and germination time of botulinus spores based on a study of approximately 37,000 sealed tube preparations which have been under inspection for from 28 to 39 months after the spores were heated.

The maximum survival times and the greatest dormancies were observed in tube preparations in which the spores were heated and incubated in broth medium which was covered with a thin layer of mineral oil.

Attention is directed to the practical application of these facts to the preservation of foods.

⁸ Harrington, Geo. T.: Jour. Agric. Res., 1916, 6, p. 20.

PRODUCTION OF MYCELIUM BY OIDIUM ALBICANS

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In a previous report¹ are detailed experiments that resulted in the production of mycelium by *Oidium albicans* in a watery extract of carrots. The following experiments were undertaken to determine what constituent in the carrot influences mycelial production.

Carrot infusion was prepared by macerating about 800 gm. of carrots in 250 c.c. of distilled water for 48 hours at room temperature. This extract was then sterilized and dialyzed through parchment paper at room temperature against 750 c.c. of distilled water until the dialysate gave a positive Molisch reaction for carbohydrates. This solution is called extract 1, and contains some of the carbohydrates and salts present in carrots.

TABLE 1
FAVORABLE INFLUENCE OF THE ASH OF CARROTS ON THE FORMATION OF MYCELIUM

Medium	Mycelial Growth
1% Wittes' peptone in distilled water.....	Yeast forms only
Equal parts of peptone water and ash solution (extract 3).....	Mycelial growth
Equal parts of peptone water and carbohydrate solution (extract 1)....	Mycelial growth
Equal parts of peptone water and protein solution (extract 2).....	Yeast forms only
Equal parts of peptone water and extracts 1, 2 and 3.....	Mycelial growth
Protein solution (extract 2).....	Yeast forms only
Carbohydrate solution (extract 1).....	No growth

The contents of the bag were then dialyzed against running tap water and tested frequently for the presence of proteins and carbohydrates. After 15 days, the contents no longer gave a positive Molisch reaction. This is extract 2.

Extract 3 consisted of the white ash of 4 carrots dissolved in 1,000 c.c. of distilled water and had a salt content equivalent to that in the usual carrot infusion which favored the formation of mycelium.

The influence of these factors is indicated in table 1. Mycelial growth appeared whenever carrot ash was added to the medium or was present in the dialysate. Mycelial growth began to appear in small rhizoid colonies adherent to the test-tube at some distance from the sur-

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¹ Draper: Jour. Infect. Dis., 1924, 34, p. 631.

face of the medium, and a very large mass of mycelium formed about one half inch below the surface. If undisturbed, the growth retained this character for weeks.

SUMMARY

Something in the ash of carrots causes *Oidium albicans* to form mycelia in peptone water, to which the ash has been added. The proteins and carbohydrates in carrots are without effect.

THE EXAMINATION OF SPOILED CANNED FOODS

I. METHODS AND DIAGNOSIS

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One of the primary functions of this laboratory is to investigate spoilage problems with view of alleviating commercial spoilage conditions. These spoilage problems, of bacterial origin, are investigated primarily to determine the cause of spoilage in canned foods, the presence of potential spoilage organisms in sound canned foods and the conditions of storage under which spoilage may occur. Information gained as to the extent of the presence of certain nonspoilage types, i. e., dormant aerobic spore-formers, was incidental to the principal purpose of investigation as the routine examination does not include an absolute sterility test of the contents from cans which have remained normal during the regular incubation period. Incubation of flat cans under favorable conditions should produce sufficient evidence to indicate the presence or absence of viable spoilage organisms.

The presence of heat resistant aerobic spore-bearing organisms that remain dormant during prolonged storage appears to have a limited significance in the study of spoilage. Several cases of this kind have been investigated, and the findings confirm, in general, those of other investigators as regards the extent of unsterility in canned foods. The problem of reduction and the ultimate prevention of spoilage in canned foods resolves itself not into a study of dormant sporing aerobes which may have survived the process, but rather the determination of the causative agents in spoilage, their distribution, the factors inducing their growth, and sterilization procedures which will be effective for their destruction.

During the last 6 years, over 600 samples have been submitted from commercial canners throughout the country for bacteriologic examination. The great majority of these were submitted, either for a diagnosis as to the cause of spoilage or for the determination of "commercial sterility" to establish the possibility of spoilage developing within the pack. The term "commercial sterility" is used in contrast to "absolute sterility" to denote the absence of organisms capable of producing spoilage under conditions of commercial manipulation. Based on field and

laboratory experience, a routine method for the bacteriologic examination of canned foods has been developed. Investigations concerning the presence of pathogens and types dormant or not readily cultivated constitute special studies requiring special technic and culture mediums.

It should be emphasized, then, that together with the results obtained by the following methods of examination, the various correlations involving the historical, bacteriologic and physical data should be considered (1) to determine the cause of spoilage in spoiled canned food (understerilization or defective containers), and (2) to determine the possibility of spoilage in sound canned foods, and under what conditions such spoilage may occur.

SPOILAGE CLASSIFIED ACCORDING TO CONDITION OF CAN AND CONTENTS

Flat Cans.—A normal can is one in which the ends are flat or curved slightly inward. This normal condition of the container is not absolute assurance of the absence of spoilage, since "flat sours" in which spoilage has occurred resulting in an increase in the acidity of the canned contents without the evolution of gas give no reliable external manifestation even when subjected to the various heat, percussion, and shake tests.

Flippers.—The can is normally flat. The "flipper" condition is indicated on percussion by the bulging of one end of the can. When the end is forced back the can should remain flat. A lack of vacuum is denoted which may be due to incipient spoilage, leakage of air, the production of hydrogen in acid products or sealing at too low a temperature.

Springers.—A springer is a can with one end bulging. Under thumb pressure the bulge flattens with simultaneous bulging of the opposite end. Cans with both ends bulged may also be considered springers, provided one of the ends can be readily flattened. According to Bigelow,¹ "Springers are due to the following causes:

a—Pressure from hydrogen generated as a result of chemical action of the acid contents on the metal of the container.

b—Imperfect closing of the can, in which the paper gasket is impervious to bacteria but admits air.

c—Overfilling of the can, in which products of heavy consistency are especially involved.

d—Sealing at too low a temperature.

e—"Dented cans."

In addition, the "springers" may be due to incipient spoilage.

Swells.—A "swell" is a can with both ends bulged. If the ends yield under thumb pressure but resist forcing to the flat condition, the term "soft swell" is used. If no impression is made by thumb pressure the can is considered a "hard swell." If there is rigid distortion of either end the can is considered "buckled." The "swell" may be due to bacterial activity in all canned foods, or in acid products to the action of the acid on the container with a resulting production of a "hydrogen swell."

¹ N. C. A. Circular 6-L, 1923.

SAMPLING

As a preliminary to the analysis on which is to be based a diagnosis as to cause of spoilage, or the presence or absence of dormant spoilage organisms, the selection of the "sample" is of utmost importance. The term is elastic and any attempt at definition must be arbitrary, yet certain basic conditions must be complied with if the resulting diagnosis is to possess any semblance of justification.

When spoilage is involved, information is desired as to its extent and whether or not one or several days' pack was affected. The time and temperature of process and the method of cooling should be learned. An examination of the seams for obvious defects should be made at the time of sampling. The percentage of cans showing apparent leakage should be noted, but cans with apparently tight seams should constitute the sample. In cases in which cans are marked, the code should be recorded. Data as to the time and temperature of storage are also to be considered.

Information concerning the retorts involved, the condition of the raw product, etc., would be valuable, but, unfortunately, it is difficult to obtain except under controlled conditions. Normal samples of the same pack should accompany the spoiled samples for purposes of comparison after incubation. Careful sampling is imperative in the event of the occurrence of "flat sour" spoilage, since it is impossible by present methods to determine externally its extent both as regards amount and distribution, whereas spoilage manifested by the swelling of the container is readily estimated.

In the absence of definite spoilage it is often desirable to determine the keeping qualities of a pack. Such a determination involves sterility tests, and is meaningless when only a few cans constitute the sample. Here the entire pack in question should be adequately sampled. As regards the size of sample, it is the custom of this laboratory to request a minimum of 24 representative cans on which to base the diagnosis.

INCUBATION OF SAMPLES

Samples of canned food are incubated only when it is desired to determine the condition of the samples, or the pack represented by them, as regards the presence of dormant bacteria capable of vegetation in the products in question. The purpose of incubation is obviously to render unnecessary the impracticable employment of mass cultures for inoculation. Virulent spoilage types should respond to this treatment,

but not organisms whose dormancy is forced by prevailing chemical (spore-formers in acid products) or physical (aerobic spore-formers, etc.) conditions.

The time of incubation appears to have been an arbitrary matter with the various investigators of canned foods. Weinzirl² incubated 7 days or longer at 37 C.; Cheyney,³ from 1 to 6 weeks with 2 weeks as the usual time, while Savage⁴ records an incubation of "several weeks."

In routine procedure it is the custom of this laboratory to incubate at 37 C. for a minimum of 10 days, and at 55 C. for from 4 to 7 days. A 10 days' incubation at 37 C. should suffice for all acid foods, but in nonacid products when the presence of facultative thermophiles, or certain slow-growing types is suspected, a longer time may be required. The 55 C. incubation applies to nonacid products only, and here, due to the absence of the retarded germination among thermophilic spores, the 4 to 7 day incubation should be sufficient. Cans are examined at frequent intervals and cultured immediately when swelled. At the end of the incubation period, all cans are tested. The significance of the findings at these temperatures will be discussed later in this paper.

TECHNIC AND SYSTEM OF CULTURES

Condition of Container.—After incubation and cooling to room temperature, the external condition of the can is classified as flat, flipper, springer, soft or hard swell. The condition of the seams is recorded, and a search for "pin-holes" made if acid products are under investigation. Any other points are noted which may have a bearing on the final diagnosis, among which are code, make of can, and any regular marks or dents on the cover or on the side seam, as these may indicate the year of packing.

Cleansing and Opening the Container.—The practice followed in this laboratory is first to thoroughly scrub the can with soap and water. A good precaution is to follow this by wiping with alcohol. The final preparation for opening consists in flaming the end to be opened. Here it is highly desirable to hold the end to be flamed above the flame as convection currents are set up inducing distribution of the heat. If the can end is flamed from above, most of the heat is retained in the upper head-space, and charring of the product occurs which may obscure the normal odor and taste. Flat cans are flamed until flipping occurs as internal positive pressure is a desirable safeguard against laboratory contamination.

There is some hazard attached to the flaming of swelled cans. This is obviated to some extent by cooling in the icebox, then thoroughly wiping with alcohol and igniting.

² Jour. Med. Res. 1919, 39, pp. 349-413.

³ Ibid., 1919, 40, pp. 177-197.

⁴ Savage, W. G., Hunwicke, R. F. and Calder, R. B.: The Bacteriology of Canned Meat and Fish, Special Report No. 11, Food Investigation Board, London, England (1920).

The spiral can-opener, set to cut a hole 1"—1½" in diameter appears best for opening. This type of opener is easily sterilized and favors rapid manipulation. In this laboratory, all operations are conducted in an easily cleaned and sterilized inoculating room, thus limiting to some extent the danger of external contamination.

Culturing.—Untapered pipets, of 4-5 mm. bore, are used in the usual culturing operation. A minimum of four 2 c.c. inoculations are made in each medium for each temperature of incubation. The methods of treatment vary with acid and nonacid products and will be considered separately.

Nonacid Products—Vegetables, Meat, Fish and Milk.—Other investigators of canned foods, Weinzi¹,² Cheyney,³ Savage, et al.,⁴ used a greater number and variety of mediums than those adopted by this laboratory, but it is to be noted that these workers were dealing with the general bacteriology of canned foods involving search for pathogens and dormant types as well as for spoilage bacteria. In this laboratory, emphasis is placed on the recovery and study of spoilage types, and it is desired to become acquainted with the organisms present from the standpoint of (1) general morphology, (2) ability to form spores, (3) relation to oxygen, (4) relation to temperature, (5) general gas and acid-producing properties in the presence and absence of oxygen, each of which has special significance.

Comparative tests have shown that special mediums employing the food to be tested as a base are unnecessary. Weinzi¹ states in this connection that starch agar could well be substituted for all special mediums. Savage and co-workers⁴ consider special mediums to be neither necessary nor helpful.

Standard plain and dextrose nutrient broths PH 7 with bromcresol purple indicator have been found entirely suitable in the routine search for aerobic and facultative anaerobic forms. For the strict anaerobes, dextrose peptic digest beef heart medium PH 7.4 stratified with petrolatum has been used with success (Stickel and Meyer⁵).

Cultures from nonacid products are incubated at 37 C. and 55 C. for at least one week.

When flat cans are to be tested for the "flat sour" type of spoilage and it is not desired to culture nonacid cans, our practice is to remove aseptically some of the food material and test in a suitable indicator solution. Bromcresol purple has been found satisfactory for testing corn, peas, lima beans, and other foods of this type, while changes in hydrogen ion concentration in the

⁵ Preparation of beef heart peptic digest liver broth (for principles involved see Holman: Jour. Bacteriol., 1919, 4, p. 149; Stickel and Meyer: Jour. Infect. Dis., 1918, 23, p. 68, and H. H. Heller: Jour. Bacteriol., 1921, 6, p. 445): (1) Slowly heat to boiling finely ground, fat-free heart, 1,000 gm., and tap water, 1,000 c.c.; adjust to a reaction of PH 8.0-8.2, then cool and carefully skim off the layer of fat which floats on the cold medium. To each liter of beef heart mash, add 2 liters of peptic digest broth.² Adjust the reaction to PH 7.2-7.4. (2) Wash clean and mince finely 5 or more large pigs' stomachs. Mince an equal amount of clean pig's or beef liver. Mix in the following proportions:

Mincd pigs' stomachs.....	400 gm.
Mincd liver.....	400 gm.
Hydrochloric acid (Baker Chemical Co.).....	40 gm.
Tap water at 50 C.....	4,000 gm.

Keep the mixture in glass or porcelain receptacles for 18-24 hours. Make biuret and also tryptophan test. When both reactions are positive, the digest is green-yellowish and contains little undigested debris. Transfer to large bottles and steam for 10 minutes at 100 C. to stop digestion. Strain the digest through cotton, or preferably store over night in the ice chest and decant after 24 hours. Warm the decanted digest to 70 C. and neutralize with sodium carbonate (twice normal solution) to litmus at this temperature. Filter the desired amount, add 0.2% dibasic potassium phosphate; adjust to PH 7.4 and mix with beef heart mash. Adjust the final reactions and sterilize for one hour at 18 lbs. of pressure. Incubate for 5 days and repeat the same sterilization for one hour at 18 lbs. of pressure.

slightly more acid products—string beans, asparagus and spinach—appear well marked in bromcresol green (Cohen⁶).

The experience of the laboratory has been such as to justify the abandonment of direct plating, reliance now being placed on plating after enrichment. The obvious objection to enrichment, i. e., the danger of overgrowth, with more than one type present is not usually valid in cases of understerilization. Here, except when grossly understerilized, it is only in an exceptional case that more than one type of spoilage organism is present. Moreover, if overgrowth would occur in the culture tubes, it probably would have taken place in the can prior to culturing. In cases of spoilage through can leaks, the flora is usually mixed, and here too it has become standardized before culturing. After enrichment in the various mediums, the condition of the original flora as regards purity should be readily ascertainable. Direct plating is objected to on the basis of unnecessary duplication of work and the danger of plate contaminations which may lead to erroneous conclusions.

Acid Products—Tomatoes and Fruits.—Spoilage types peculiar to these foods appear to be limited to the yeast and to the nonsporing, mesophilic, facultative anaerobic, acid-loving bacteria. Here, special mediums having tomato juice as a base have been found to be of value in separating spoilage types from organisms unable to grow in acid substrate. The liquid medium tomato dextrose broth consists of tomato juice and nutrient broth in equal parts with the addition of 1% dextrose, sterilized with or without pressure. The solid medium is made by adding 3% agar to the tomato dextrose broth and sterilizing for 1 hour in the Arnold sterilizer. In both of these mediums, due to the normal acidity, sterilization under atmospheric pressure is adequate, and it is essential for tomato agar since pressure sterilization induces hydrolysis and renders this medium useless.

Procedure with these foods, therefore, resolves itself into the inoculation of aerobic tubes of tomato dextrose broth, with the parallel inoculation of nutrient sugar broths, to be incubated at 35 C. to 37 C. After enrichment the plating and purification of tomato spoilage types, the tomato agar is desirable.

Microscopic Examination of Contents.—Smears made direct from the canned product often give valuable information in the final correlation of results. Here, the ordinary carbol-fuchsin or gentian violet stains usually give the results desired. Record is made of the apparent condition of the flora, whether pure or mixed, the presence of non-heat-resistant forms, of which the cocci are typical, and the presence of spores.

Physical Examination of Contents.—The appearance and odor of the product is noted as soon as practicable after culture. Differences in the type of spoilage in individual cans of the same sample are recorded as these have considerable bearing in the final analysis. It is not the practice in this laboratory to taste spoiled canned foods.

Examination of Cultures.—Cultural observations are made on both aerobic and anaerobic tubes at 37 C. and 55 C., special note being made as to the presence of gas and acid, and the temperature at which they are formed. A microscopic examination of positive cultures gives evidence as to the general morphology of types present, and the degree of purity of the original food contaminant.

When bacteriologic evidence indicates understerilization, cultures are purified by any of the common methods. After assurance as to the ability of the purified culture to form spores (in nonacid products), it is set aside for immediate or later classification.

⁶ Abst. Bacteriol., 1923, 7, p. 3.

In the event of an exhaustive study, the heat resistance of the spore is determined by the method of Esty and Williams.⁷ Finally, an attempt is made to reproduce spoilage by inoculation into sterile samples of the particular food from which it was isolated.

With acid foods, since thermophiles and spore-forming bacteria do not appear to enter as causative spoilage agents, observations are confined to the single temperature and the heat resistance tests apply only to vegetative forms.

B. BOTULINUS IN COMMERCIALLY CANNED FOODS

B. botulinus (types A, B and C) is the only known pathogenic spore-former significant in causing spoilage of canned foods with the production of a preformed toxin. It has been definitely established that spores of *B. botulinus* can grow and produce its toxin in a large variety of nonacid canned foods, such as most of the canned vegetables, meats, milk and fish. It has also been definitely shown that *B. botulinus* spoilage in canned foods is caused by incomplete sterilization. It is difficult to predict with any degree of accuracy the percentage of containers of commercially canned foods which harbor viable spores of *B. botulinus*, but in view of the small number of outbreaks that have been reported in comparison to the total number of cans packed, and the extreme rarity with which the organism has been recovered from suspected containers, it is safe to conclude that the number of dangerous cans is negligible. From all available sources, it is quite evident that the commercial methods have been generally sufficient, and understerilization as far as *B. botulinus* spores is concerned is rare. Consequently, in view of the undisputed rarity of the occurrence of botulinus spoilage in commercially canned foods, it does not appear practicable to include a search for its presence as a routine procedure. In the event of suspicion as to the presence of the toxin or viable spores of *B. botulinus*, the method of examination as proposed by Dubovsky and Meyer⁸ is recommended.

PHYSICAL EXAMINATION OF CANS⁹

The application of methods employed in the routine examination for can defects requires technicians thoroughly conversant with the essential indications of seam abnormalities. Hermetically sealed cans are of two types—the sanitary or open top can, and the hole and cap can. By sanitary can is meant a can the ends of which are attached by means of

⁷ *Ibid.*, 1924, 34, p. 516.

⁸ *Jour. Infect. Dis.*, 1922, 31, pp. 527 and 534.

⁹ The methods used in the physical examination of cans were not developed in this laboratory, but have been used for several years by those particularly interested in the manufacture of cans.

a crimped double seam, the only solder used being that along the side seam. Either a paper gasket or a rubber composition gasket is used between the crimped seams to make a tight seal. This type of can has, except with milk and meat, practically superseded the hole and cap can in which the ends are attached by solder. A general outline of the methods used in the testing of cans follows.

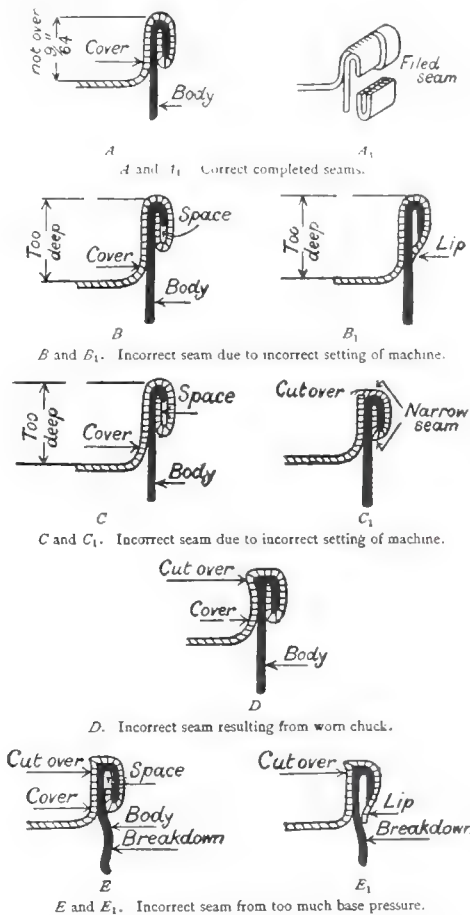


Fig. 1.—Sanitary cans; double seams. By courtesy of the American Can Co.

The can is first examined for the presence of any obvious points of leakage such as cut-overs (fig. 1), cracked seams, defective side seams, and punctures. After the contents are removed, the cans are carefully washed and boiled one hour in water, using no alkali or other additional cleansing agent, to remove if possible any food material which may have been forced into minute leaks.

After boiling, the water is removed, using a suction tube to dispose of the last few drops. The cans are dried at about 100 C. for one hour, and the opening made to remove material for cultures is sealed by a soldered cap.

The can is punctured through the cap with an apparatus (attached to a source of air pressure) equipped with a pressure gage (fig. 2). After immersion in water, examinations are made for leaks at definite pressures.

Number 2 cans are tested at intervals of 5 pounds up to 25 pounds pressure; number 2½ cans up to 15 or 20 pounds; and number 10 cans up to 10 or 15 pounds, the difference in maximum pressure used being due to the ability of the smaller sized cans to withstand greater pressure.

There is some degree of air filtration when paper gaskets have been used in sealing the can, and the examination resolves itself into a search for excessive leakage, either general or local. Compound gaskets (rubber composition) should completely exclude the egress of air. Particular attention should be paid to side seams and butt joints (junction of side and end seams), also to any dented points of the body near the seams.

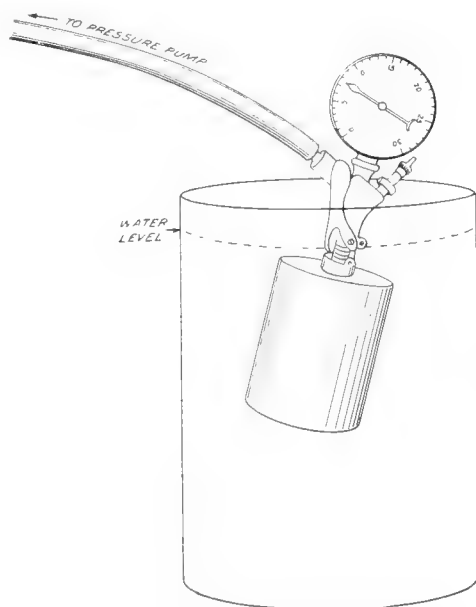


Fig. 2.—Pressure pump.

After pressure testing, the seams are filed and torn apart to expose possible defects. A cross-section is filed at two or three points on each end seam, and the condition of the body and end hooks noted under a small magnifying glass. With the use of a pair of pliers, the end seam is pulled down so that it stands at right angles to the body of the can. This gives an opportunity to note the smoothness of the rolling, the position of the gasket, and the length of the end hook. It is also best to pull apart and examine the side seam laps at either end of the can. If the side seam is suspected, it should be pulled apart along its entire length to note the condition of the solder.

When examining cans with soldered seams (hole and cap can), it is not necessary to file a cross-section. The seams are merely torn apart to note whether the solder is continuous or broken. Figure 1 illustrates diagrammatically normal and certain abnormal seam aspects met with in the examination of the sanitary can.

DIAGNOSIS

As the result of a study of spoilage, the diagnosis should state the cause of spoilage as being due to understerilization or to the leakage of bacteria through seam defects. In a study of sterility, the diagnosis should state the condition of the samples as regards the possibility of future spoilage in the pack represented by them. By means of the foregoing methods for the examination of canned foods, special points of significance are established for determining whether the spoilage was due to understerilization of the canned contents, or to the leakage of bacteria through defective seams. The diagnostic value of these methods is shown in the correlation of the experimental results obtained from the bacteriologic and physical tests with the available field data bearing on the history, method, and condition of the pack from which samples were submitted. Special emphasis is placed on the importance of certain features in interpreting discordant results sometimes obtained by the different tests. The items of significance will be discussed essentially in the order of the description of methods.

1. A comprehensive consideration of reliable field data regarding the condition of the pack, process, method of cooling and storage conditions may furnish presumptive evidence as to the cause of spoilage or of any potential spoilage. If a cursory examination of the seams of representative swelled cans reveals obvious defects, the swelling appears to be due to faulty containers, and in the majority of cases needs only microscopic confirmation.

Information regarding the condition of the pack, the extent of spoilage and how it is distributed throughout the pack is invaluable. Spoilage in the form of swells or flat sours in cans with apparently tight seams when confined to a certain lot in a day's run or a certain retort in which a large majority of the cans are spoiled, is indicative of understerilization. This may be due either to some radical error in processing as to time or temperature, or to a greater contamination of the raw product resulting either from some unusual handling in the preparation or to a delay in the processing. Moreover, spoilage distributed throughout the season's pack is usually due to faulty double seaming or defective cans, but may sometimes be accounted for on the basis of understerilization, indicating that a borderline process may have been used.

2. A "swell" may result from either understerilization or leakage. Therefore, in the absence of obvious defects, the fact that a can is swelled is of no immediate diagnostic significance.

On the other hand, the observation that the container is flat, coupled with a subsequent discovery of "flat sour" spoilage (nonacid products) is strongly indicative of understerilization as it is hardly possible that bacteria gaining entrance through defects would consist entirely of non-gas producing types and extensive leakage should result in the swelling of a considerable number of cans affected.

3. The development of either "flat sours" or "swells" during incubation at 37 C. or 55 C. is direct evidence of understerilization, provided that a sufficient time has elapsed between the time of packing and incubation to allow the vegetation of organisms capable of development at ordinary storage temperature. Spoilage confined to the incubation at 55 C. gives added data denoting improper cooling after processing or prolonged storage at temperatures above 37 C.

The behavior on incubation of a sample made up of cans from a supposedly sterile pack should permit a qualitative and quantitative estimate of the condition of the pack. Spoilage at 37 C. gives basis for recommendations as to the storage temperature of the pack or even reprocessing, according to the time of incubation necessary to cause spoilage. If spoilage occurs at 55 C. but not at 37 C., it would appear sufficient to recommend that storage temperatures be limited to below 37 C.

4. On microscopic examination of direct smears from nonacid foods, the presence of the non-heat-resistant coccus forms or the observation of a mixed flora with or without coccus forms is strong presumptive evidence of faulty double-seaming or some other can defect. On the other hand, when an apparently pure culture of bacilli is observed with some indication or the actual presence of spores, understerilization is strongly suggested. The presence of a mixed flora when confined to bacillary types may denote either understerilization or defective containers. However, the microscopic findings obtained from the original material are merely indicative and are to be used in comparison with those from bacterial cultures.

5. The odor and appearance of the spoiled product is generally noted as a matter of record and as basis for comparison when it is desired to reproduce the spoilage with organisms isolated. The only general point of immediate significance would be lack of uniformity in types of spoilage among cans of the same sample, which is considered somewhat presumptive of spoilage through leakage.

6. The bacteriologic examination of suspected food is of utmost importance, and contributes most in the analysis of the findings. Here is obtained the main evidence as to the cause of spoilage. By means of sufficient nutrients and a favorable temperature, the causative agent is isolated, and the spoilage can be readily reproduced. Observations made during the investigation of more than 600 spoilage cases permit of the following conclusions so far as the bacteriologic examination is concerned. In this connection, the presence of dormant aerobes manifested by an occasional culture tube showing growth is not considered as a spoilage index.

A. Nonacid Products.—The presence of impure cultures of rod-shaped bacteria capable of causing spoilage is indicative of some can defect or a gross error in the sterilization. Occasionally, spoilage cases have been investigated in which more than one type of resistant spoilage bacteria has been found, but usually even in such cases the cause of spoilage can be readily determined by the inoculation of pure cultures, representing the types present, into sound cans of the same food and employing a favorable environment for growth. However, in cases in which different types of organisms such as a flat sour associated with a gas former are present, the question is which type of spoilage will develop more readily under existing conditions. One type usually predominates in many cases inhibiting the activity of, and sometimes destroying, other types.

The presence of a pure culture of heat resistant bacteria capable of reproducing the spoilage in canned food is definitely attributable to understerilization. The presence of impure cultures containing non-heat-resistant gas-forming types, such as certain cocci and nonsporing bacilli, is definite evidence that the organism entered the container through leakage.

Diagnostic significance has been attached to the presence of certain predominant spoilage types in commercially canned foods, which will be discussed in a subsequent paper.

Understerilization in spoiled, nonacid canned foods usually has been found to be due to thermophilic spore-bearing facultative anaerobes in the case of flat sours and to obligate anaerobes in the case of swells. In this laboratory, organisms failing to show growth below 40 C. over a period of several months are considered obligate thermophiles, and those growing readily both at 37 C. and 55 C. facultative thermophiles. This temperature requirement varies for different foods, but the terminology holds as defined for specific substrates. For example, organisms have been studied which acted as facultative thermophiles in some foods and as obligate thermophiles in others.

No typical flat sour mesophilic bacterium has been isolated, but occasionally mesophilic spore-bearing organisms have been found to be responsible for the spoilage of nonacid foods with the production of swells. *Sporogenes*-like organisms have been recovered from canned sardines, peas and corn, although such cases are rare in the latter two foods. The majority of the mesophilic organisms in canned foods belong to the group of ordinary sporing aerobes, the spores of which remain dormant, and as such are unable to germinate unless air gains access to the food.

B. Acid Products.—Flat sours have not been encountered in acid products, nor has any spoilage directly attributable to spore-forming organisms been observed. Organisms have been isolated by Savage¹⁰ and by ourselves from spoiled acid products which failed to show gas production in a variety of mediums in glass but which when reinoculated into the can produced a "swell" with the production of CO₂. Accordingly, in the examination of swelled acid products, a non-gas-forming organism isolated through a medium based on the food examined should be studied with reference to the influence of the metal container before definite conclusions can be drawn as to its identity as the causative agent in the original swelling. This question is receiving further study and will be discussed in a subsequent paper.

In the spoilage of acid products, bacteriologic findings are limited in their significance by the fact that no specific differences have been observed between the floras of understerilized and leaky cans, so in these products a first-hand knowledge of packing conditions (method of cooling) and a well based interpretation of the condition of the seams is essential.

7. The determination of the heat resistance of organisms supposed to be responsible for spoilage resulting from understerilization often gives valuable confirmatory evidence, especially if the resistance is determined in the original unspoiled product. Heat resistance results obtained in the food juice according to the method of Esty and Williams⁸ must be interpreted in the light of such factors as the rate of heat penetration in the specific food, and the number of spores heated. Even though the resistance is tested in cans of sound material, the inoculum as regards the number of spores heated must be seriously considered.

¹⁰ Savage, W. G., and Hunwicke, R. F.: Canned Fruit, Special Report, No. 16, Food Investigation Board, London, England, 1923.

TABLE 1

THE EXAMINATION OF SPOILED CANNED FOODS: A SUMMARY OF SIGNIFICANT FEATURES AND THEIR RELATION TO THE CAUSE OF SPOILAGE

Significant Features	Nonacid Products			Spoilage from Leakage of Can	Acid Products	
	Spoilage from Understerilization		Spoilage from Leakage of Can		Spoilage from Understerilization	Spoilage from Leakage of Can
	"Flat Sours"	"Swells"				
Condition of container	Flat	Swelled	Swelled	Swelled	Swelled	Swelled
Microscopic examination of direct smears from can	Rods, with or without apparent spore formation, usually apparently pure cultures	Rods, with or without apparent spore formation, usually apparently pure cultures	Evident impure cultures usually containing cocci forms	Apparently impure cultures ranging from coccoid bacilli to very long rod forms.* Yeast ?	Apparently impure cultures ranging from coccoid bacilli to very long rod forms. Yeast	Apparently impure cultures ranging from coccoid bacilli to very long rod forms. Yeast
Physical examination of contents	Usually sloppy in appearance; normal or sour odor	Fermented appearance; sour or putrid odor	Fermented appearance; sour or putrid odor; sometimes variations in appearance and odor in different cans of the same sample	Fermented appearance; sour, cheesy or alcoholic odor	Fermented appearance; sour, cheesy or alcoholic odor	Fermented appearance; sour, cheesy or alcoholic odor
Cultures	Aerobic and anaerobic growth at 37 C. and 55 C. or at 55 C. alone; no gas formation; usually evidence of spore formation in agar; microscopic examination should check direct smears	Growth anaerobically with gas at 37 C. or 55 C.; or both 37 C. and 55 C.; if aerobic growth occurs without gas formation at 37 C. and 55 C., or 55 C. alone the presence of a flat sour type is also suggested; microscopic examination should check direct smears	Usually gas anaerobically and aerobically at 37 C. with or without growth at 55 C.; microscopic examination should show mixed culture and show presence of non-heat-resistant types	Growth in special acid mediums at 37 C., with or without gas production; if no gas is produced, reinoculate in sound cans to determine ability to produce swell; microscopically pleomorphic forms with possible presence of yeast	Growth in special acid mediums at 37 C., with or without gas production; if no gas is produced, reinoculate in sound cans to determine ability to produce swell; microscopically pleomorphic forms with possible presence of yeast	Growth in special acid mediums at 37 C., with or without gas production; if no gas is produced, reinoculate in sound cans to determine ability to produce swell; microscopically pleomorphic forms with possible presence of yeast
Seam examination	No leaks on pressure testing; seams and general condition of container good	With "hard swells" leaks on pressure testing may be due to straining; seams should give indication of original sound condition	Leaks on pressure testing; seams or general condition of can faulty	No leaks on pressure testing; seams and general condition of container good	No leaks on pressure testing; seams and general condition of container good	Leaks on pressure testing; seams or general condition of can faulty

* This apparent impurity of culture is usually found to be due to the extreme pleomorphism evidenced by the group of organisms most significant in the spoilage of acid products.

If a few spores of the suspected organism capable of reproducing the spoilage are found to be relatively heat resistant, definite information as to a certain process is available. If, on the other hand, it takes a large number of these spores to become sufficiently resistant to have survived a certain process, the original spoilage may be accounted for on the basis of an error in sterilization as to time or temperature, or is indicative of the condition of the raw material. The efficiency of a certain process should be determined on standardized spore suspensions of pure cultures in sound canned material instead of on portions of the spoiled material, since in the latter case the formation of certain metabolic products may result in the failure of vegetative forms to sporulate or even in their destruction. The influence of these products may also seriously affect the time necessary to sterilize. Hence, heat resistance determinations made on spoiled material may be misleading or even lead to erroneous conclusions.

8. The container may be considered as tight, doubtful, or leaky on the basis of the pressure test and seam examination, but, as previously stated, this examination should be conducted only by those possessing intimate knowledge regarding the nature of normal and abnormal seams. Seam examination, however, is essential to a complete chain of evidence confirming, in general, the microscopic and bacteriologic findings. In the event of disagreement between the physical and biologic test, i. e., a pure culture of a heat resistant thermophilic organism in defective containers, or non-heat-resistant types in apparently tight containers, it is imperative to consider all available data. If further examination by supplementary methods, or from more samples, reveals consistent, biologic results, it is believed that these deserve the greater consideration.

The significant features in their relation to the cause of spoilage as being due either to understerilization or defective containers are summarized in table 1.

SUMMARY

The spoilage of canned food is classified according to the condition of the can and contents as flat ("flat sour"), flippers, springers, and swells.

Methods for the bacteriologic examination of canned foods to determine the cause of spoilage or "commercial sterility" involve in non-acid products (vegetables, meat, fish, and milk), a systematic study of aerobic and anaerobic cultures incubated at 37 C. and 55 C., and stained preparations both of the original canned material and the cultures, together with a physical examination of the container to detect defects, as disclosed by pressure testing and the filing and stripping of the seams. Similar tests are applied to acid foods with the exception that only aerobic cultures in dextrose and tomato dextrose broth at 37 C. are studied. Special culture mediums prepared from food materials are unnecessary in testing nonacid foods.

The following significant features are considered in their relation to the cause of spoilage as being due either to understerilization or to faulty

containers: (*a*) field data regarding the general condition of the pack, methods employed, storage conditions, and, in case of spoilage, the extent and nature of the trouble; (*b*) flat sours, as indicative of understerilization; (*c*) the development of "swells" or "flat sours" during incubation at 37 and 55 C., and especially at 55 C.; (*d*) microscopic findings of direct smears from nonacid foods; (*e*) odor and appearance of the spoiled product; (*f*) bacteriologic evidence, especially so far as pure cultures of predominant spoilage types are present; (*g*) heat resistance results, and (*h*) the condition of the container.

In spoiled nonacid foods, the presence of pure cultures of thermophilic spore-bearing facultative anaerobes in the case of "flat sours" and obligate anaerobes in the case of "swells" denotes understerilization, frequently accompanied by improper cooling after processing.

In spoiled nonacid foods, the presence of impure cultures, usually containing coccus forms developing aerobically and anaerobically at 37 C., with or without growth at 55 C., denotes spoilage from faulty containers. Gas development in aerobic cultures at 37 C. is further evidence of some can defect. In such cases, variations in appearance and odor may be noted in individual cans of the same sample.

In spoiled acid foods (fruits and tomatoes), the presence of apparently impure cultures, ranging from coccoid bacilli to very long rod forms and occasionally yeasts developing in tomato dextrose broth at 37 C., with or without gas production in sound containers, denotes understerilization, whereas the same flora in leaky cans, evidenced by pressure testing and seam examination, denotes spoilage from faulty containers.

PRODUCTION OF LOCAL IMMUNITY BY MEANS OF DIPHTHERIA TOXIN

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The occurrence of local immunity was thought to exist for many years, but was not definitely studied as such until Besredka,¹ published his work on immunity against the paratyphoid bacillus by means of "galled" vaccine given by mouth. The possibilities of this method were apparent, and it was put to the test as often as opportunity arose, and with favorable results, according to reports in the French literature from time to time. Not only is the method efficacious in preventing the disease, but its administration is also attended, in the great majority of cases, with no systemic reactions such as occur with the old method. In the few cases showing reactions, the symptoms are slight and consist of fever, diarrhea with colic, headache and vomiting.

From paratyphoid fever, the investigations turned to other enteric diseases, such as typhoid, cholera, dysentery and undulant fever, in most of which the same methods were followed (ingestion of ox gall tablets followed by tablets containing the organisms previously killed by heat), with similar favorable results.² In infections peculiar to the skin, particularly those caused by the staphylococcus, streptococcus and *B. anthracis*, the intradermal use of these organisms in the form of vaccines, or of the various products of growth, frequently gave a high degree of skin immunity.³

LOCAL IMMUNITY AGAINST *B. DIPHTHERIAE*

The present methods of immunization, by the injection of antitoxin or by toxin-antitoxin mixtures, have proved of great value in the control of diphtheria. No method is perfect, however, if a simpler and less dangerous one can be devised. With this idea in mind, the following experiments were made to determine whether a mucous membrane could be immunized against diphtheria by the use of minute harmless doses of toxin.

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¹ Ann. de l'Inst. Pasteur, 1918, 32, p. 193; 1919, 33, p. 557.

² Ibid., 1919, 33, p. 301 and p. 882.

³ Ibid., 1921, 35, p. 422; 1922, 36, p. 562; Comp. rend. Soc. de biol., 1923, 88, p. 1273; 1923, 89, p. 7 and p. 506.

Babes⁴ inoculated the conjunctiva in rabbits; exudates formed in 24 hours; some died, some developed paralysis. Several subsequent observers showed that membranes may be formed by inoculations into the injured cornea. Coppez⁵ investigated the influence of diphtheria toxin on the eye. He used one instillation, and, failing to obtain any pathologic effects, concluded that the toxin cannot produce lesions if the corneal epithelium remains intact. Morax and Elmassian⁶ instilled toxin diluted 1:5 every 3 minutes for 8 hours into the conjunctival sacs of rabbits. All of the effects of diphtheria toxin were produced, including the formation of false membrane. Some of the animals died of acute toxemia, others developed cachexia. Römer⁷ showed that when a toxin was repeatedly instilled into the conjunctiva of one eye of an animal that conjunctiva at a certain time was able to neutralize a given dose of toxin, whereas the other unimmunized conjunctiva possessed no such power.

Since our work was done, Zoeller⁸ reports that he was unable to produce a local immunity in the skin of guinea-pigs by the intradermal administration of doses of diphtheria toxin just sufficient to produce a local reaction and a small scar. However, Zoeller⁹ was able to prevent the infection in the eyes of guinea-pigs that had been treated with bile and then inoculated with diphtheria bacilli according to the method of Zoeller and Manonssakis¹⁰ by the subcutaneous use of Ramon's anatoxin.

EXPERIMENTS

Because of the difficulty in performing experimental work on the mucous membranes of the nose and throat of small laboratory animals, the more readily accessible mucous membrane of the eye was chosen. I first found that lesions could be produced with regularity only on the injured conjunctiva. For example: inoculations of a loopful of a heavy suspension of virulent *B. diphtheriae* were made twice daily over a period of 4 days, on the palpebral conjunctiva of the upper lid, previously excoriated with a sharp needle. Twenty-four hours after inoculation, a slight local swelling occurred; in 48 hours, this had involved the upper lid, and sometimes the lower, and began to have a gelatinous appearance. In 72 hours, the edema had spread to the tissue about the eye, and opacities began to appear in the cornea. It was not determined whether these opacities were due to membrane. At about this stage, large amounts of purulent material containing diphtheria bacilli were discharged from the eyes, which closed with a total loss of sight and a disappearance of the eyeball. During the active discharging stage, the guinea-pigs appeared toxic, and 2 died as a result, showing enlarged suprarenals with moderate degrees of injection. If the uninjured conjunctiva is inoculated in the same way, no infection results.

Also as a preliminary step, a diphtheria toxin of low potency (1 c.c. killed a 250 gm. guinea-pig within 36 hours) was produced by allowing the organisms to grow with pellicle formation on a sugar-free broth medium over a period of 8 days. Vaccination was begun on 2 series of 6 pigs weighing about 250 gm. each. One series was treated with the toxin, and the other with a washed

⁴ Quoted by Nuttall and Graham-Smith; *Diphtheria*, 1913, p. 276.

⁵ *Rev. gen. d'ophth.*, 1897, 10, p. 197.

⁶ *Ann. d. l'inst. Pasteur*, 1898, 12, p. 210.

⁷ Quoted by Nuttall, and Graham-Smith, *Diphtheria*, 1913, p. 514.

⁸ *Compt. rend. Soc. de biol.* 1924, 90, p. 1147.

⁹ *Ibid.*, p. 1400.

¹⁰ *Ibid.*, p. 1399.

salt solution suspension of the bacilli heated to 70 C. for one hour. Twice daily 1 drop was instilled into the left eye of each guinea-pig. No local changes occurred. At the end of the 6th day (12 drops), both series began to show general symptoms, namely, quietness and stiffness of the hind legs. Instillations were stopped for one day and then resumed. On the night of the 8th day (after each had received 14 drops), 2 of the toxin guinea-pigs and 1 of the vaccine guinea-pigs died. At necropsy, they showed enlarged suprarenals. Four days later (12 days after vaccination had been started), inoculation with living bacilli was begun on the 4 remaining toxin treated guinea-pigs, on the 4 guinea-pigs treated with vaccine and on 4 untreated guinea-pigs, as controls.

As in the experiments to produce lesions in fresh guinea-pigs the lids were excoriated and inoculated at 24-hour intervals with 1 loopful of a heavy suspension of the bacilli, over a period of 4 days. In the controls (table 1), the

TABLE 1

SHOWING THE EFFECT OF PLANTING DIPHTHERIA BACILLI ON THE SCARIFIED CONJUNCTIVA OF GUINEA-PIGS. CONTROLS FOR TABLES 2 AND 3

	Second Inoculation, 24 Hours after First	Third Inoculation, 48 Hours after First	Fourth Inoculation, 72 Hours after First	Later Observations
1. Left eye.....	No lesion	Gelatinous edema; slight corneal opac- ity; slight discharge	Inoculations stopped	Recovered slowly
2. Left eye.....	No lesion	Slight edema	Edema in- creased; cor- neal opacity; discharge	Edema in- creased; lids closed; opac- ity marked; loss of eye
3. Right eye.....	No lesion	Marked edema	Discharge	Discharge in- creased; eye closed; condi- tion poor; died 24 hours after last inoculation
4. Right eye.....	No lesion	No lesion	Slight edema	Edema marked; slight opacity; discharge; re- covery with scarring

inoculations were carried only to the point of corneal opacity. Necropsy on one of the controls showed enlarged, injected suprarenals. In the guinea-pigs treated with toxin and in those treated with dead bacilli, no lesions developed; 24 hours after the inoculations were stopped, the controls and the animals treated with the toxin (table 2) showed positive cultures from the eyes; those previously vaccinated with the suspension of dead organisms (table 3) showed negative cultures. At the end of 2 weeks, negative cultures were secured from the guinea-pigs treated with toxin, as well as from the vaccinated guinea-pigs. Further observations over a period of 2 months showed no new developments; in other words, what appeared to be a definite local immunity was present in the conjunctiva of the animals treated with toxin and in those treated with a suspension of dead organisms.

TABLE 2

RESULTS OF PLANTING DIPHTHERIA BACILLI ON THE SCARIFIED CONJUNCTIVA OF GUINEA-PIGS
PREVIOUSLY TREATED WITH TOXIN OF UNKNOWN STRENGTH

	Second Inoculation, 24 Hours after First	Third Inoculation, 48 Hours after First	Fourth Inoculation, 72 Hours after First	Later Observations
1. Left eye.....	No lesion	No lesion	Slight edema	Edema not increased; no further changes
2. Left eye.....	No lesion	No lesion	No lesion	No lesion
3. Right eye.....	No lesion	No lesion	No lesion	No lesion
4. Right eye.....	No lesion	No lesion	No lesion	No lesion

TABLE 3

RESULTS OF PLANTING DIPHTHERIA BACILLI ON THE SCARIFIED CONJUNCTIVA OF GUINEA-PIGS
PREVIOUSLY TREATED WITH A WASHED SUSPENSION OF DEAD BACILLI

	Second Inoculation, 24 Hours after First	Third Inoculation, 48 Hours after First	Fourth Inoculation, 72 Hours after First	Later Observations
1. Right eye.....	No lesion	No lesion	No lesion	No lesion
2. Right eye.....	No lesion	No lesion	No lesion	No lesion
3. Left eye.....	No lesion	No lesion	No lesion	No lesion
4. Left eye.....	No lesion	No lesion	Edema and opacity	Edema in- creased; dis- charge; gen- eral condition good; recovery complete

TABLE 4

RESULTS IN GUINEA-PIGS PREVIOUSLY TREATED WITH TOXIN OR VACCINE AND LATER
INOCULATED WITH LIVING BACILLI

	Second Inoculation, 24 Hours after First	Third Inoculation, 48 Hours after First	Fourth Inoculation, 72 Hours after First	Later Observations
Vaccine, guinea-pig 1; right eye immunized, left eye inoculated	Slight local reaction	No change	No change	No change
Vaccine, guinea-pig 2; right eye immunized, left eye inoculated	Slight local reaction	Slight increase of local reaction	No change	No change
Toxin, guinea-pig 4; right eye immunized, right eye inoculated	Slight local reaction	No change	No change	No change
Toxin, guinea-pig 1; left eye immunized, right eye inoculated	Slight local reaction	Slight increase of local reaction	Edema further increased; slight systemic symptoms	Recovery complete

An attempt was made to determine the duration and extent of this immunity. The guinea-pigs which had been immunized about 2 months previously were divided into 2 groups, each group having 2 pigs immunized by each method. Of these 2 groups, one was inoculated with living bacilli in the immunized or opposite eye, and in the case of the other group, on the abraded surface of the belly wall (tables 4 and 5). Inoculation into the eye was carried out

TABLE 5
RESULTS IN GUINEA-PIGS PREVIOUSLY TREATED WITH TOXIN OR VACCINE AND LATER
INOCULATED ON THE BELLY WALL

	Second Inoculation, 24 Hours after First	Third Inoculation, 48 Hours after First	Fourth Inoculation, 72 Hours after First	Later Observations
Vaccine, guinea-pig 4.....	Scratches healed; scabs soft; indura- tion marked	Pus about wound; sub- cutaneous tis- sues softening	Area of in- volvement less; scabs raised and hardened	Condition of wound slowly improved; slight systemic symptoms de- veloped; later recovery com- plete
Vaccine, guinea-pig 3.....	Same as above	Infiltration of subcutaneous tissues; some pus	Scabs raised and hardened	Wound slowly cleared; syste- mic symptoms developed; later recov- ery complete
Toxin, guinea-pig 2.....	Same	Scabs hard- ened; no in- filtration	No change	Local edema extensive; systemic symp- toms marked; recovery slow but complete
Toxin, guinea-pig 3.....	Same	Slight infiltra- tion of sub- cutaneous tissues	Scabs hard but easily raised; edema marked	Died on night following 4th inoculation; suprarenals enlarged, with slight injection

as before, one loopful of a heavy suspension of living virulent bacilli being placed on the excoriated conjunctiva at 24-hour intervals for 4 days. The results showed some little differences between those treated with toxin and those vaccinated with a killed suspension of organisms. In the latter, only a slight transitory local reaction, consisting of redness and slight swelling, occurred after the 3rd inoculation; while in the former this local reaction increased to a partial closure of the eyes, and in one of the guinea-pigs to definite systemic symptoms—weakness and stiffness of the hind legs. Within one week complete recovery ensued in all the guinea-pigs.

In the experiments to determine the extent of the immunity, 2 controls were studied. In these, the belly wall was shaved and scratched with a sharp knife, then a loopful of a heavy suspension of the bacilli rubbed on the surface once every 24 hours for a period of 4 days. After the first inoculation, the scratches appeared healed, but a slight area of redness surrounded them. The scabs were lifted slightly and another loopful of the suspension rubbed over the surface. After the 2d inoculation, definite collections of pus appeared beneath the skin, surrounded by areas of edema and induration. These signs

increased, and both guinea-pigs died on the night following the last inoculation, with definite lesions at necropsy. The guinea-pigs previously vaccinated were treated in a like manner (table 5). In all, scabs formed over the scratches, which were hard but easily raised. In one, there was suppuration followed by edema, but to a much less extent than in the controls. In all, edema developed, and at the end of the 4th inoculation all showed more or less

TABLE 6
SHOWING THE EFFECT OF PLANTING DIPHTHERIA BACILLI ON THE SCARIFIED CONJUNCTIVA OF GUINEA-PIGS PREVIOUSLY VACCINATED WITH SHICK TOXIN OF FULL STRENGTH

	Second Inoculation, 24 Hours after First	Third Inoculation, 48 Hours after First	Fourth Inoculation, 72 Hours after First	Later Observations
1. Right eye.....	Weakened condition due to intestinal disorder; inoculations stopped	Recovered
2. Right eye.....	Slight local edema	Increase of edema	No change	Recovery
3. Left eye.....	Same as above	No change	No change	Recovery
4. Left eye.....	Same	No change	Increase of edema	Recovery
5. Left eye.....	Same	No change	No change	Recovery

TABLE 7
SHOWING THE EFFECT OF PLANTING DIPHTHERIA BACILLI ON THE SCARIFIED CONJUNCTIVA OF GUINEA-PIGS PREVIOUSLY VACCINATED WITH SCHICK TOXIN DILUTED 1:3

	Second Inoculation, 24 Hours after First	Third Inoculation, 48 Hours after First	Fourth Inoculation, 72 Hours after First	Later Observations
1. Right eye.....	Slight local edema	No change	No change	Recovery
2. Right eye.....	Same as above	No change	No change	Recovery
3. Left eye.....	Same	Increase in edema	Edema same; cornea cloudy	Recovery
4. Left eye.....	Same	No change	No change	Recovery
5. Left eye.....	Same	Slight increase in edema	No change	Recovery

systemic symptoms. One of the guinea-pigs died on the 5th day with all the lesions typical of diphtheria. All the others subsequently recovered.

Another experiment to determine the strength of toxin necessary to produce immunity was carried out at the same time as the foregoing experiments. Two series of 5 pigs each were treated with toxin: (a) one of such strength as that used in the Shick reaction (approximately 1/50 M L D to the size of drops used), (b) the other with this toxin diluted 1:3. These guinea-pigs

were given 1 drop a day for 7 days. No local symptoms followed these instillations. The results of 4 inoculations of living bacilli into the eyes of the guinea-pigs, one week following vaccination with the full strength Shick toxin were much the same as those previously obtained with the unstandardized toxin guinea-pigs. (Compare tables 2 and 6.) In all of them, after the initial inoculation, a slight edema developed, but this did not increase on subsequent inoculations, and had entirely disappeared 2 days after the experiment. In those treated with the diluted toxin, the edema, which appeared after the first inoculation, increased slightly in 2 of the animals after the 2d treatment, but stopped here (table 7). In one, the edema increased slightly after each inoculation and showed a just visible opacity of the cornea at the end of the experiment, which later entirely disappeared.

SUMMARY

Lesions of definite character may be regularly produced on the previously excoriated conjunctiva of the guinea-pig by the local application of living virulent diphtheria bacilli.

Dilute diphtheria toxin and washed suspensions of *B. diphtheriae*, killed by heat, in limited doses produce no ill effects when placed on the uninjured conjunctival surface, but if the instillations are repeated too often general intoxication results.

Previous treatment of the conjunctival surface with correct amounts of toxin or suspension of killed diphtheria bacilli protects the animal against subsequent inoculation of the injured conjunctiva with virulent bacilli. This local immunity was shown to be of at least 2 months' duration. The local immunity is apparently accompanied by a low degree of general immunity.

BLOOD CHANGES IN B. WELCHII INFECTION

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In a previous article,¹ I showed that a chronic infection with *B. welchii* had been produced in rabbits, characterized by mild anemia, loss of weight, convulsions and diarrhea. Following intrasplenic inoculation of cultures of *B. welchii*, blood changes appear in from 6 to 48 hours and continue as long as the animal lives (2 days to 5 months). Following inoculation elsewhere, e. g., subcutaneous, blood changes do not appear for 3 weeks, but once present, continue until death.

Erythrocyte Count.—The anemia is usually chronic and mild, but in exceptional cases is subacute or acute, and profound. The red count shows irregular variations. The greatest depression occurs in the early part of the disease and is always followed, sooner or later, by a compensatory rise which no doubt represents the response of a healthy hemopoietic system. Examination of the longest surviving animals shows that the red count, though falling slowly, is falling progressively. A terminal rise is explained by the presence of a severe dehydrating diarrhea (table 1).

Hemoglobin Estimation.—The average rabbit shows a maximum fall of 42% from its normal reading. The steepest downward gradient occurs during the early part of the disease. Subsequently, although fluctuations may occur, a general level considerably below normal is maintained. The original normal level is never regained except artificially, due to terminal diarrhea.

The color index shows irregular variations. In the presence of definite anemia, the index is less often below, than at or above, 1. The absence of a stable type of erythrocyte depression explains the absence of a stable index level. In index estimations, the same conventional standards were used as clinically in man (see table 1).

Leukocyte Count.—In cases in which inoculation is made intrasplenically, the leukocytes show an initial fall, followed by a marked rise, after which the count subsides to nearly normal, only to rise again more slowly and less high. In cases inoculated subcutaneously, there is usually a gradual rise, followed by varying fluctuations. The differential counts

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are irregular. Absolute computations of the polymorphonuclear neutrophils and the lymphocytes show that both types of cell are subject to numerical variation, but especially the former. None of the cases show marked eosinophilia or basophilia (table 1).

Morphologic Changes.—Moderate poikilocytosis is frequently present, but never marked. Nucleated red cells appear only when an acute, severe anemia is in progress. At such times, stippling may be present, and polychromatophilia pronounced. The basic staining requires a much less marked anemia for its production and is more frequently present than absent throughout the course of the disease.

TABLE 1
BLOOD DATA, RABBIT 261*

	Red Blood Cells	Hemo-globin		Differential White Blood Cells							Absolute Numbers	
		%	Index	White Blood Cells	Poly-morpho-nuclear Neutrophils	Lym-phocytes	Baso-phils	Large Mono-nuclears	Eosino-phils	Myelo-cytes	Poly-morpho-nuclear Neutrophils	Lym-phocytes
Normal	6,912,000	67	1	7,800	39	50.5	2.5	6.5	1.5	0	3,042	3,989
Jan. 11	6,776,000	66	0.9	9,800	50.6	38.7	6.0	2.7	2.0	0	4,959	3,793
Jan. 17	7,376,000	64	0.9	12,000	23.0	52.0	8.5	8.0	8.5	0	2,760	6,240
Jan. 30	5,632,000	58	1.1	7,600	65.0	25.0	1.0	4.5	4.5	0	4,940	1,900
Feb. 6	5,732,000	48	0.8	7,000	49.0	40.5	3.0	3.0	4.5	0	3,430	2,835
Feb. 13	5,904,000	54	0.8	9,000	61.0	29.5	7.5	1.0	1.0	0	5,490	2,655
Feb. 20	5,712,000	41	0.8	9,800	47.5	45.5	5.0	1.0	1.0	0	4,655	3,969
Mar. 6	4,976,000	48	0.9	8,800								
Mar. 11	4,824,000	43	0.8	7,600								

* On Jan. 5, this rabbit received subcutaneously 1 c.c. of a glucose muscle, anaerobic culture of a fairly virulent strain from human stools.

Anisocytosis is, however, the most constant blood change in these experiments. After it once appears, it invariably remains until death, being most pronounced when anemia is most marked and least pronounced when anemia is least marked and the general condition best. Anisocytosis of the erythrocytes of the general circulation usually appears within 6 to 8 hours following intrasplenic inoculation, and within 3 weeks following subcutaneous inoculation. It is so constant that its absence is an indication that inoculation has failed to produce infection. The ordinary smear examinations reveal that anisocytosis is constantly present from a certain period after inoculation until death. This fact is substantiated and elaborated by micrometer measurements. By making 500 cell measurements on smears taken before inoculation and at various intervals afterward, it is found that the diameter changes conform to the following plan:

1. Within from a few hours to 2 days after intrasplenic inoculation, there appears 1% or 2% of microcytes and a smaller percentage of macrocytes, while the average diameter slightly decreases.

2. With the next 3 or 4 days, the macrocytes increase (e. g., to 5%), the microcytes decrease (e. g., to 0.5%) and the average diameter increases above normal (e. g., by 0.5μ).

3. After about 2 weeks, one of two courses may be followed: (a) The average diameter may again drop below normal with a preponderance of microcytes over macrocytes, and remain thus till death. (b) The average diameter may remain above normal with preponderance of macrocytes over microcytes until just before death.

4. In any case, just prior to death, the average diameter usually falls below normal, an effect presumably due to the lethal accumulation of toxin.

5. Following subcutaneous inoculation, the changes are the same as described, but, as stated, they appear less promptly.

The tables illustrate these facts graphically. In rabbit 214 (table 2), it will be noted that 27 hours after intrasplenic inoculation the average diameter has fallen from 6.11 to 6.01, and the microcytes are 4 times as numerous as the macrocytes, whereas, on the 4th day after inoculation, the average diameter has increased to 6.64, and the macrocytes are 9 times as numerous as the microcytes. Sixteen days after inoculation, the average diameter, though still greater than normal, has fallen 0.4, and the microcytes are again more numerous than the macrocytes. Toward the end of the disease, the balance still favors the microcytes, and the average diameter is less than normal. In rabbit 262, a study of table 3 shows a case in which microcytes and macrocytes remained equally numerous. In rabbit 261, a study of table 4, shows a case in which macrocytes preponderated until near the end. These 3 cases are selected at random and are typical of all the rest.

Striking as these results are, even more marked effects are occasionally obtained, as in the case of rabbit 4193, the blood data being given in tables 5 and 6. Within 5 days after subcutaneous inoculation, a tremendous hemolysis had occurred, comparable to that observed when the toxic filtrate of *B. welchii* is given intravenously. The marked rise between Feb. 11 and 14 is due to a profuse diarrhea which ushered in death.

The tables make clear the fact that the majority, 76.4%, of all the erythrocytes in the circulation have become macrocytes. There is no

explanation for the extreme blood picture developed in this exceptional case. Culture of tissues at death gave positive, pure cultures of *B. welchii* from the skeletal muscles and tongue, and sterile cultures from other tissues. Whether a heavy muscle infection with *B. welchii* explains

TABLE 2

MICROMETER MEASUREMENTS OF RED CELLS IN RABBIT 214, INJECTED INTRASPLENICALLY, OCT. 26, 1923, AT 6 P. M.

Diameter of Cells in Mikrons	Normal	Oct. 27, 9 p.m.	Oct. 30	Nov. 12	Dec. 26
1.89	1
2.06	1
2.58	1	2
3.26	..	1
3.27	2	2
3.44	..	3	..	4	7
3.61	..	2	..	4	10
3.78	..	1	..	1	2
3.95	2	2
4.13	1	2
4.30	..	5	..	5	14
4.47	..	1
4.64	..	4
4.82	..	5	3
4.99	1	1	2	5	12
5.16	27	64	10	24	33
5.33	32	33	15	15	27
5.50	52	63	17	28	26
5.68	20	26	..	6	5
5.88	21	25	14	33	20
6.06	72	71	49	57	66
6.23	80	34	44	39	43
6.40	52	17	38	35	32
6.57	55	18	64	39	37
6.75	41	34	58	70	76
6.92	40	56	86	89	60
7.09	6	8	45	22	8
7.27	..	4	15	6	7
7.44	..	0	2	1	3
7.61	2	0	..
7.79	1	2	9	5	..
7.96	..	1	6	1	..
8.30	..	1	2	2	1
8.48	..	2	8	2	1
8.65	..	2	8	1	..
8.82	1
9.00	..	1	1
	500 cells	500 cells	500 cells	500 cells	500 cells
Smallest diameter...	4.99	3.26	4.82	2.58	1.89
Largest diameter...	7.79	9.00	9.00	8.65	8.48
Average diameter...	6.11	6.01	6.64	6.24	6.05
Microcytes.....	4.4%	0.6%	4.0%	8.6%
Macrocytes.....	1.4%	5.2%	1.2%	0.4%

it or not, is uncertain. Nine other rabbits inoculated at the same time with the same amount of the same culture showed much less striking effects and an infection less specifically muscular at death.

Incidentally, the leukocytes show certain morphologic changes as well. The polymorphonuclear neutrophils show a decrease in average diameter of 1.26 mikrons, and, at the same time, 20% of cells larger

than any normally occurring, while the average number of nuclei per cell increase, from 2.81 to 3.77, but there is no disturbance in the size, arrangement, number or staining qualities of the granules. The lymphocytes show an increase of average diameter of 1.05 mikrons, and 8.8% of cells larger than any normally occurring.

TABLE 3
MICROMETER MEASUREMENTS OF RED CELLS IN RABBIT 262 INJECTED SUBCUTANEOUSLY

Diameter of Cells in Mikrons	Normal	Feb. 13, 1924 5 Weeks	Feb. 28, 1924 7 Weeks
2.23.....	..	1	..
2.92.....	1
3.09.....	..	1	..
3.44.....	..	3	..
3.61.....	..	5	..
3.78.....	2
3.95.....	1	3	..
3.96.....	2
4.13.....	1
4.30.....	..	12	5
4.47.....	1	17	4
4.64.....	..	8	3
4.82.....	..	13	10
4.99.....	2	26	35
5.16.....	30	98	84
5.33.....	20	54	38
5.50.....	23	33	38
5.68.....	23	9	8
5.88.....	40	32	55
6.06.....	71	69	74
6.23.....	61	18	34
6.40.....	34	8	19
6.57.....	58	14	19
6.75.....	63	30	30
6.92.....	68	38	32
7.09.....	4	5	3
7.27.....	1
7.79.....	..	1	3
7.96.....	..	1	..
8.65.....	..	1	..
	500 cells	500 cells	500 cells
Smallest diameter.....	3.95	2.23	2.92
Largest diameter.....	7.27	8.65	7.79
Average diameter.....	6.192	5.64	5.74
Microcytes.....	2%	0.6%
Macrocytes.....	0.6%	0.6%

The anisocytosis of the erythrocytes is apparently a specific effect of *B. welchii*, since the intrasplenic injection of various other organisms (for technic see Cornell¹) grown on the same medium, e. g., hemolytic streptococci, hemolytic staphylococci, pneumococci, *B. coli*—does not cause any morphologic disturbance. Etherization, opening the abdomen and ligating the tip of the spleen cause no morphologic change, nor is any produced by injecting into the spleen certain chemical and physical agents, e. g., 95% alcohol, boiling water, 1% butyric acid, nor by the operation of splenectomy.

TABLE 4
MICROMETER MEASUREMENTS OF RED CELLS IN RABBIT 261, INJECTED SUBCUTANEOUSLY

Diameter of Cells in Mikrons	Normal	One Month after Injection	Six Weeks after Injection
2.58.....	..	1	..
3.10.....	..	3	1
3.27.....	1
3.44.....	..	1	2
3.61.....	9
3.78.....	..	5	..
3.95.....	..	1	..
4.13.....	2
4.30.....	1	2	9
4.47.....	..	1	4
4.64.....	2	3	3
4.82.....	2	3	6
4.99.....	2	9	16
5.16.....	67	42	51
5.33.....	55	30	28
5.50.....	69	29	28
5.68.....	27	9	4
5.88.....	40	28	21
6.06.....	63	36	59
6.23.....	43	33	24
6.40.....	33	30	22
6.57.....	20	29	27
6.15.....	40	63	59
6.92.....	25	83	80
7.09.....	10	23	28
7.27.....	1	15	5
7.44.....	..	5	2
7.61.....	1
7.79.....	..	5	8
7.96.....	..	1	..
8.13.....	..	4	..
8.30.....	..	1	..
8.48.....	..	2	..
8.65.....	..	3	..
	500 cells	500 cells	500 cells
Smallest diameter.....	4.3	2.58	3.10
Largest diameter.....	7.27	8.65	7.79
Average diameter.....	5.54	6.21	5.7
Microcytes.....	2.22%	3%
Macrocytes.....	4.2%	2.22%

TABLE 5
RABBIT 4193, INOCULATED SUBCUTANEOUSLY WITH 0.25 C.C., 24 HOUR AEROBIC CULTURE ON ROBERTSON'S MEAT OF STRAIN C, FROM THE BONE MARROW AT DEATH OF RABBIT 280

	Hemo- globin	Erythro- cytes	Leuko- cytes	Index	Smear
Normal.....	59	6,288,000	6,400	...	Normal
Feb. 5, 10 a.m.	14	1,296,000	17,400	0.9	Tremendous anisocytosis, nucleated reds, crenations, many polychromatophilic macrocytes
Feb. 5, 5 p.m.	16	512,000	13,400	2.8	Same as last
Feb. 5, 8 p.m.	16	1,264,000	11,800	0.9	Same with a few megaloblasts
Feb. 6, noon.....	26	1,560,000	18,000	1.5	Same as last
Feb. 6, 8 p.m.....	26	1,776,000	7,000	1.3	Same except fewer nucleated reds
Feb. 7, noon.....	29	2,032,000	11,400	1.2	Anisocytosis, megaloblasts stippling
Feb. 8, 9 p.m.....	36	3,016,000	7,400	2.0	Macrocytes less large; stippling
Feb. 11, 1924.....	36	3,288,000	11,000	1.0	Anisocytosis, less
Feb. 14, 1924.....	71	5,616,000	6,600	1.8	Anisocytosis, less
Feb. 14, 1924. Death					

If before injecting 1 c.c. of *B. welchii* culture, it be mixed with an equal volume of *B. welchii* antitoxic horse serum and allowed to stand at room temperature for 2 hours, no such prompt blood picture develops,

TABLE 6
MICROMETER MEASUREMENTS OF RED CELLS OF RABBIT 4193, INOCULATED SUBCUTANEOUSLY,
JAN. 31, 1924

Diameter of Cells in Mikrons	Normal	5 Days after Injection
2.41.....	..	1
2.58.....	..	1
3.44.....	2	1
3.61.....	..	7
3.78.....	..	1
3.95.....	..	2
4.13.....	..	1
4.30.....	..	3
4.47.....	..	4
4.82.....	..	2
4.99.....	2	2
5.16.....	36	6
5.33.....	49	5
5.50.....	49	11
5.68.....	25	..
5.88.....	36	3
6.06.....	106	21
6.23.....	77	7
6.40.....	50	8
6.57.....	19	12
6.75.....	16	6
6.92.....	33	32
7.09.....	..	32
7.27.....	..	15
7.44.....	..	12
7.61.....	..	2
7.79.....	..	59
7.96.....	..	14
8.13.....	..	11
8.30.....	..	9
8.48.....	..	35
8.65.....	..	106
8.82.....	..	21
9.00.....	..	11
9.17.....	..	2
9.34.....	..	1
9.52.....	..	30
9.69.....	..	7
9.86.....	..	3
9.98.....	..	3
10.15.....	..	5
10.32.....	..	12
10.49.....	..	1
12.04.....	..	1
	500 cells	500 cells
Smallest diameter.....	3.44	2.41
Largest diameter.....	6.92	12.04
Average diameter.....	5.943	8.45
Microcytes.....	0.4%
Macrocytes.....	76.4%

although a delayed one, equally marked, appears after a period of from 10 days to 2 weeks. This inhibition of the effect of culture is not duplicated by other antitoxic or antimicrobial serums, e. g., antipneumococcus, antimeningitis, diphtheria antitoxin, tetanus antitoxin, nor by normal horse serum. These facts indicate that this phenomenon of

anisocytosis is due to the toxin of *B. welchii*. But it is not necessary to have any performed toxin in the culture injected, since a fresh suspension of organisms made from the colonies on an anaerobic blood-agar plate produces the same prompt morphologic disturbance as does the Robertson's meat culture. The effective toxin is therefore probably that formed *in vivo* by the growth of the organism in the spleen, and elsewhere by dissemination.

The most effective sites of inoculation are the spleen and the marrow, although the muscle is sometimes equally good. The liver, the peritoneum and the ear veins were found least suitable, since inoculation into these may cause no perceptible blood changes even after weeks. From these statements it might be suspected that certain organs bear an essential relationship to the production of the blood picture. Such is not the case. The anisocytosis is apparently an effect of *B. welchii* toxin directly on the erythrocytes of the general circulation, without the intermediate agency of any organ or substance. If the two external jugulars of a rabbit under urethane be completely tied off from the circulation, as living tubes of blood, and into one be injected a sufficient amount of a sufficiently potent toxin, the smears from this vein after an hour will show definite anisocytosis, whereas the control vein which received no injection will show a normal smear. Moreover, defibrinated rabbit blood treated *in vitro* with sufficient toxin shows, after an hour's incubation, the same phenomenon. Washed erythrocytes of the rabbit, similarly treated, show the same phenomenon. In all three cases just described, the anisocytosis is a phase preliminary to complete hemolysis, since all the erythrocytes eventually disappear if enough toxin is present. Nor is this *in vitro* phenomenon peculiar to the blood of the rabbit. It occurs also with the blood of dogs, mice, cats, guinea-pigs, man, and presumably other mammals, although for different animals' blood, differences in the amount of toxin required will be found—a fact probably depending on the varying amounts of natural protective substances present in the serums.

Nature of the Anisocytosis.—The microcytes have the appearance in stained smears of contracted, well-pigmented erythrocytes, while the macrocytes have the appearance of relaxed cells. In smears from infected rabbits, the macrocytes frequently take the basic stain, but never in smears from the *in vitro* preparations. In smears from rabbits suffering the acute infective anemia and in smears from *in vitro* preparations many macrocytes with pale centers as well as shadow forms are seen. Whether

this type of hemolysis with a preliminary phase of anisocytosis is specific for *B. welchii* toxin, is unknown. Saponin, while strongly hemolytic, does not produce, even in lethal doses, any morphologic disturbance of the red cells. The intravenous injection of a large volume of distilled water produces a transient waterlogging of the erythrocytes which is not comparable with the phenomenon described in this paper.

DISCUSSION

Some essential component substance of the exotoxin of *B. welchii* is responsible for the anisocytosis found so constantly in this experimental disease. In the infected animal, this substance given off by the micro-organism affects, no doubt, in some way, every cell of the animal. The evidence adduced to date indicates that the toxic action responsible for blood changes is not necessarily directed chiefly against the hemopoietic tissues, but rather that it affects directly the red cells of the general circulation. This property of the exotoxin of *B. welchii* should lend a somewhat novel direction to our studies of anisocytosis-producing agents in general. Indeed, this experimental blood picture bears so close a resemblance to that seen in pernicious anemia as to create a fresh interest in Herter's well-known hypothesis regarding *B. welchii* as the cause of that disease. The strains used in these experiments were isolated mostly from the stools of patients suffering from Addison's anemia, but investigation shows that strains from wounds produce the same picture. Blood changes said to resemble those of pernicious anemia have been produced by injecting ricin, pyridine and fecal extracts, but in the present experiments the causative agent is a living micro-organism. Since it has been shown¹ that this organism is capable of producing a chronic infection, it is not unreasonable to imagine that pernicious anemia might be a chronic infection by *B. welchii*.

CONCLUSIONS

Rabbits infected with *B. welchii* show a chronic anemia of varying degree.

The blood picture presents, as its chief point of interest, the constant phenomenon of anisocytosis.

This anisocytosis is due to the direct effect of the toxin of *B. welchii* on the erythrocytes of the general circulation.

HABITAT OF *B. HISTOLYTICUS* IN HUMAN INTESTINE

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Among the rarer organisms which were found to be concerned in wound infections during the World War was *B. histolyticus*. This bacillus, unique as regards its pathogenesis, was first described by Weinberg and Sequin¹ in 1916. They were able to isolate it from 8 cases of gaseous gangrene, and seem to have been the only investigators of that period to do so. It was surmised to be an intestinal organism and to find its entrance into wounds from polluted soil. Although the British Medical Research Committee in their report² stated that it has been obtained from soil, the first definite information in regard to these points were the reports of Petersen and Hall² on its isolation from arable soil in California and of Hall³ on its recovery from a human stool specimen.

As regards the levels in the human intestine at which this organism may be found, whether it passes through the intestine as a mere "casual" or under certain conditions may find the intestinal environment so favorable as to permit active growth and presumably the production of its toxin, believed by Weinberg to be of an extracellular nature, are questions in regard to which there seems to be no published information. It is the purpose of this communication to present rather complete data in regard to some of these points as determined by the bacteriologic study of material from two human intestines.

This study was made possible through the courtesy and cooperation of Dr. John W. Draper who performed colectomy operations on these patients in the service of Dr. Henry Cotton at the State Hospital for the Insane, Trenton, N. J., and through the assistance of my associate, Dr. J. W. Churchman, who collected the material from various segments at the time of operation. It constitutes a part of a rather extensive investigation in intestinal bacteriology and the absorption of bacterial products which is being conducted in this laboratory.

One of these cases was that of a boy (H), aged 14, with severe epilepsy, who had suffered from frequent attacks since childhood, sometimes as many as 10 a day. This patient was also markedly constipated. The other patient

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¹ La Gangrene Gaseuse, 1917.

² Quoted from Petersen, E., and Hall, I. C.: Proc. Soc. Exp. Biol. & Med., 1923, 20, p. 503.

³ Proc. Soc. Exp. Biol. & Med., 1923, 21, p. 198.

(C) had a psychosis. He had improved somewhat, but prior to the operation had developed an extreme condition of constipation amounting almost to an obstruction, as feces were found impacted all the way up to the ileocecal valve. Both of these patients had been on an ordinary diet for some time previous to the operations. For about 4 days preceding the operation, they had undergone a period of catharsis with castor oil and also had been given enemas.

In collecting the material for bacteriologic study, the bowel, as soon as possible after removal, was segmented by ligatures at the ileocecal valve at the distal end of the cecum and at the distal end of the transverse colon. These segments were then opened and material removed from the lumen, together with scrapings from the mucosa, specimens thus being collected from the lower ileum, cecum, transverse colon, and the sigmoidal region. The specimens were placed in labeled test tubes, and these in turn were packed with ice in a large insulated jar. In this way the material was kept at freezing temperature until it could be examined, which was at times not for 24 hours or longer, owing to the distance from the place of operation. It is not believed, however, that any loss occurred in numbers or types of bacteria during this interval.

The methods employed in the general bacteriologic examination of these specimens will be described elsewhere, and merely those concerned in the isolation of *B. histolyticus* and the estimation of its relative numbers are given here.

Graded dilutions of the material were made in a series of 5 tubes, so that in tube 5 the material in tube 1 was diluted 1:10,000. The density of the suspension in tube 1 was made as close to an accepted standard as possible. Films prepared from no. 1 suspension were stained with the Kopeloff and Beerman⁴ modification of the Gram stain and examined microscopically. For the cultivation and detection of the spore-bearing anaerobes, cooked meat medium, with a reaction of P_H 7.2 was employed. These tubes were made anaerobic by boiling just before seeding and sealing with a petrolatum cap, as described by Kahn.⁵ Two tubes were seeded respectively with 1 c.c. each of the numbers 1 and 5 dilutions, and 3 tubes with the same amount from dilutions 1, 3 and 5, respectively, heated at 80 C. for 10 minutes. These cultures were incubated for about 10 days at 37 C. and then kept at room temperature for observation over a prolonged period for detection of balls of tyrosin which are indicative of the presence of *B. histolyticus*.

In case H, examination of stained films, prepared from the ileum material, showed large numbers of bacteria with some gram-positive rods resembling morphologically *B. histolyticus*, but these were greatly outnumbered by other types. The tube of cooked meat medium seeded with heated suspension 2 of the ileum material after 24 hours' incubation showed a moderate amount of gas, and digestion of about 9% of the meat. After 3 days, the meat had assumed a dark, dull red color and a more or less disintegrated appearance. After 8 days, approximately 36% of the meat column was digested, and in 2 weeks, marked tyrosin production was in evidence as indicated by the balls of white crystals scattered throughout the meat. Subsequent identification of the spore-bearing anaerobes in this culture revealed the presence of *B. welchii*, *B. sporogenes* and *B. histolyticus*. The gram-stained cecal film from this case showed a moderate number of gram-positive rods, but none with spores. The no. 5 dilution of the heated (80 C.) material, however, when seeded in 1 c.c. amount into cooked meat medium under anaerobic conditions produced what was apparently a pure culture of *B. histolyticus*. There was a moderate pro-

⁴ Proc. Soc. Exper. Biol. & Med., 1922, 20, p. 71.

⁵ Jour. Med. Res., 1922, 43, p. 155.

duction of gas; the meat assumed a dull, dark red color, and after 8 days was digested to the extent of about 24%, with a later appearance of tyrosin crystals. This finding justified the assumption that *B. histolyticus* outnumbered other types of spore-bearing anaerobes in the cecum and were present to the number of about 10,000 per c. c. of the no. 1 suspension. As was the case for the ileum, *B. histolyticus* was here associated with *B. welchii* and also probably with *B. sporogenes*. Similar cultural tests with material from the colon of case H indicated that *B. welchii* was predominant among the spore-bearing anaerobes, although not present in very large numbers. Characteristic colonies in deep agar plants suggested also the presence of *B. sporogenes* and *B. histolyticus*, but the predominance of *B. welchii* did not permit isolation of the former types. The cultures prepared with the sigmoid material yielded evidence of the presence of *B. welchii* only; at least, this organism overgrew whatever other types might have been present.

The findings with case C were similar to those with case H, except that there seemed to be a much greater number of gram-positive spore-bearing types of bacteria in the ileum of the former case. Certain of these resembled morphologically *B. histolyticus* or *B. sporogenes*, and others *B. tertius*. The general picture gave the impression that *B. histolyticus* was multiplying in this locality, as many clumps of gram-positive rods resembling this organism were seen. Cultures from the ileum in cooked meat medium gave reactions which indicated the predominance of *B. histolyticus*, such as disintegration and digestion of the meat column to the extent of 25% in 7 days, the development of a dark, dull red color, but no foul odor. Although balls of white tyrosin crystals did not appear for about 3 weeks, subcultural tests showed a pure culture of *B. histolyticus*. Microscopic examination of the cecal material revealed gram-positive rods resembling *B. histolyticus*, but none with spores. There were 1 or 2 free spores to a field. Cooked meat medium seeded with this material, heated at 80 C., gave *B. histolyticus* growth with the no. 3 dilution, but no growth with no. 5. *B. welchii* was also found. Microscopic examination of material from the colon showed few gram-positive rods, only 1 or 2 to a field, whereas free spores were unusually numerous with an average count of 11 per field. This finding seemed to indicate that the gram-positive rods in the vegetative stage in the cecum found conditions in the colon so unfavorable, possibly because of lack of nitrogenous food material, that they were forced into the spore stage. The cultures in cooked meat medium showed that *B. welchii* was strongly predominant among the spore-bearing anaerobes in this region, as a practically pure culture developed in the tube seeded with the no. 5 dilution, whereas the presence of *B. histolyticus* was indicated by the development of tyrosin in no higher dilution than no. 1. This

was confirmed by subcultures. The small amount of mucus which constituted the material from the sigmoid region was negative for *B. histolyticus*.

In comparing the findings in these 2 cases, a marked similarity is revealed as regards the levels of the intestine at which *B. histolyticus* found conditions most favorable. In both it was present in the ileum to at least 6 inches above the ileocecal valve, and in one of the cases it was the predominant spore-bearing anaerobe in that locality. In the cecums also there was evidence of its predominance and active growth, whereas in both cases the colon seemed to offer unfavorable conditions for *B. histolyticus* and to permit the overgrowth of another representative of this group, *B. welchii*.

These *B. histolyticus*-like organisms were isolated in pure culture from the ileum and cecum in both of these cases and subjected to comparative study with 3 stock strains, all of which came originally from the Pasteur Institute. They conformed in all essential respects to these type strains. The organisms were gram-positive rods with oval, subterminal to central spores. Free spores were not found in cultures 24 to 48 hours old, as is so markedly the case with *B. sporogenes*, but practically all were still associated with the rods. Cooked meat medium is affected in a characteristic way by the growth of this organism. No gas or the merest trace is to be observed under the petrolatum cap after 24 hours' incubation. The meat takes on a rather dark, dull reddish color, and shows a progressing degree of digestion. With the 7 strains isolated from these cases, the degree of digestion of the meat after 24 hours ranged from 0.2 to 0.7 cm., whereas with the stock strains it amounted to 0.1 to 0.2 cm. After from 2 to 4 days, gas production had increased considerably, owing to proteolysis. In 12 days, the height of the meat column was lowered through digestion from 1.5 to 1.8 cm., whereas with the stock strains the decrease amounted to 1.0 to 1.2 cm. Thus it appeared that the intestinal strains possessed the greater powers of digestion for meat. White balls of tyrosin crystals were first in evidence in from 7 to 12 days with most of these intestinal strains, although in one instance they did not appear until after 20 days. Tyrosin production in this form is a specific characteristic for *B. histolyticus*. These intestinal strains also acted on milk in a characteristic way, forming a soft, mushy clot which underwent marked digestion after 3 days without gas production, becoming practically complete in 20 days. Gelatin was liquefied rapidly

by all the strains. Weinberg and Sequin,¹ Kendall, Day and Walker,⁶ Kahn⁵ and Hall⁷ all agree that *B. histolyticus* is not a fermenting organism. The test substances used in this determination for these intestinal strains were glucose, saccharose, lactose, salicin, xylose and inulin. With none of these was there production of gas or other evidence of fermentation.

As is well known, *B. histolyticus* produces a striking and peculiar lesion when injected into the muscle tissue of laboratory animals. This consists of a rapid proteolysis of the tissue, preceded by hemorrhages due to the action of the toxin on the vascular walls. When injected into the thigh muscles, the digestion may be so complete as to lay bare the bone but without gas production. There is apparently no other organism capable of producing such a lesion, and the test is, accordingly, diagnostic. Strains isolated from the ileum in both cases, C and H, were inoculated into the thigh muscles of guinea-pigs. The culture used was a 24-hour growth in casein digest, peptone, meat infusion broth, P_H 7.2. With strain H, the dosage was 0.1 and 0.5 c.c. Both animals developed typical lesions. With the smaller dose, the leg appeared swollen after 24 hours with slight subcutaneous hemorrhages, but without a break in the skin. The swelling and hemorrhage increased until after 3 days the typical lesion developed with digestion of the skin and muscle tissue along the inner side of the leg and up toward the abdomen to such a degree that the bones of the pelvic region were revealed. The animal receiving the larger dose was found dead on the 4th day. The strain tested from case C was found considerably less virulent. An injection of 0.5 c.c. of the culture produced in 24 hours a moderate local swelling with slight subcutaneous hemorrhages but without any digestion of the skin. After 3 weeks, the animal appeared perfectly normal. One c.c. of this strain, however, caused death in 48 hours, with extensive hemorrhagic proteolysis of the tissues at the site of injection. We have not as yet encountered an avirulent strain of this organism. All of our strains isolated from 4 human intestines proved virulent, although differing somewhat in degree. Hall⁸ has reported failure to produce typical lesions with the so-called "Jablons-Lister" strain, but a recent test of the representative in our collection has proved it to be fully virulent. It is perhaps worthy of note that of the strains

⁶ Jour. Infect. Dis., 1923, 30, p. 1.

⁷ Proc. Soc. Exper. Biol. & Med., 1923, 20, p. 503.

⁸ Proc. Soc. Exper. Biol. & Med., 1923, 20, p. 501.

isolated from these 2 human intestines, those showing the greater virulence were recovered from the case exhibiting by far the more serious symptoms.

The failure of investigators heretofore, except in the one instance noted above, to find *B. histolyticus* in specimens from the human stool would seem to warrant the inference that this organism rarely occurs in the human intestine. Kahn,⁹ in this laboratory, has recently completed a study of the spore-bearing anaerobes as they occurred in specimens from the stools of 72 persons, 60 of whom exhibited symptoms more or less definitely referable to the intestine, and did not encounter *B. histolyticus* in any instance. Since the completion of his study, however, we have isolated *B. histolyticus* from specimens from the stools of 2 patients, one suffering from a chronic diarrhea associated with marked anemia and the other from a case of nonspecific ulcerative colitis. In spite of the few instances in which this organism has been isolated from human fecal material, it is my belief that examinations of material from the lower small intestine and the cecum, if it were feasible to make them, from persons suffering from intestinal derangements would not infrequently reveal this organism. Failure to find it in stool specimens may well be due to overgrowth by other organisms of the same group in the colon. The peculiar nutrient requirements of this bacillus may account for its apparent localization in the lower ileum and cecum. As has been observed, *B. histolyticus* cannot utilize carbohydrates but must depend entirely on nitrogenous substances for its energy. Such being the case, it naturally would be restricted as far as growth is concerned to those levels of the bowel where the food residue had not yet been deprived by absorption of nitrogenous matter in a form which it could utilize and where other conditions favor bacterial life, that is to say, to the lower ileum and cecum. It is probable also that stasis in the colon, which was a pronounced feature in both of these cases, favors its growth at higher levels.

It is not known whether *B. histolyticus* is confined to the human intestine or occurs also in that of lower animals. Judging from its metabolic characteristics, such as its inability to utilize carbohydrates and the powerful proteolytic ferment which it elaborates, it would seem probable that this organism finds conditions most favorable in the intestines of carnivorous animals, or, at least, in species, as in man, in which meat generally forms a part of the diet. In the recently reported suc-

⁹ Jour. Infect. Dis., 1924, 35, p. 423.

cessful isolation from feces by Hall,³ the specimen came from a man whose diet contained about 100 gm. of meat a day and also an abnormally large quota of sugar, and in the two cases reported here the diet embodied a moderate amount of meat.

Although obviously *B. histolyticus* becomes implanted in the human intestine through contaminated food, my findings would seem to indicate, as has been stated, that occasionally at least it may not merely pass through the intestine in an inert state but may undergo active multiplication at certain levels of the intestine. Further evidence in support of this view is furnished by the fact that the specimen from the stool of one of these patients (H) examined by M. C. Kahn about 5 months later still showed its presence. As the colon with about 6 inches of the ileum had been removed at operation, this finding itself provided fairly conclusive evidence that the habitat of *B. histolyticus* extended well up in the small intestine in this case. Granting, then, that this organism may multiply at these levels, there is certainly a possibility that there occurs an absorption of its toxic products particularly under conditions of stasis in the large bowel. That such bacterial products may be absorbed has been established through the findings of Tenbroeck and Bauer,¹⁰ who demonstrated tetanus antitoxin in the blood of persons harboring *B. tetani* in the intestinal tract. In the case of *B. histolyticus*, it is probable that the amount of its toxic or proteolytic principle absorbed is small, and that the body becomes protected, in some measure at least, through the production of an antibody. On the other hand, there is also a possibility that the absorption of this substance over long periods of time has a cumulative harmful effect on certain organs. Although Nasta¹¹ has concluded that this proteolytic ferment exhibits an affinity almost exclusively for striated muscle, Beckwith and MacKillop¹² have recently shown that the effects produced by the injection of 2 c.c. of a 24-hour glucose broth growth into the gluteal muscle of a rabbit are not confined to the site of inoculation, but histologic examination of the liver, spleen, kidney, lung, suprarenal and heart tissues reveal "profound degenerative changes." This included the destruction of epithelial cells and the disintegration of connective tissues.

The findings reported here as regards the association of *B. histolyticus* with 4 human intestines, together with that of Hall's successful isolation from a human stool specimen and from soil, would seem to

¹⁰ Jour. Exper. Med., 1923, 37, p. 479.

¹¹ Compt. rend. Soc. de biol., 1922, 87, p. 279.

¹² Jour. Med. Res., 1924, 44, p. 311.

afford an adequate explanation of the source of infection of war wounds with this organism. It is indeed rather surprising that so few cases were noted during the World War. A possible explanation may be found in the supposition that this bacillus does not occur in the intestines of herbivorous animals and only rarely in the intestines of men in normal health; accordingly there would be little likelihood of infection of soil in the areas of combat.

SUMMARY

The recovery of *B. histolyticus* from the ileocecal region of 2 persons is reported. One had a severe and long-standing epilepsy, and the other chronic constipation with marked stasis in the cecum and colon. In addition, the organism was isolated from specimens from the stools of 2 other persons suffering from abnormal intestinal conditions.

Material from various intestinal segments was obtained at the time of colectomy operations on the first two patients. In both of these cases, *B. histolyticus* was recovered from the lower ileum, but in largest numbers from the cecum. There was some evidence of its active growth at these intestinal levels. It was observed also in the colons, but was greatly outnumbered in that locality by *B. welchii*.

It seems probable that the peculiar nutrient requirements of this bacillus account for its predominance among the spore-bearing anaerobes in the ileocecal region of these two persons.

The strains isolated from these 4 cases exhibited cultural and pathogenic characteristics typical for *B. histolyticus*. The strain from the epilepsy case was particularly virulent.

The occurrence of this organism in the lower ileum and cecum suggests the possibility of absorption of its toxic products.

These findings, together with those of Hall, provide an adequate explanation of the source of infection of war wounds with *B. histolyticus* through soil polluted with human feces.

A PRECIPITATING AND NEUTRALIZING SCARLATINAL ANTISTREPTOCOCCUS HORSE SERUM

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In the course of immunologic studies, and the injection into animals of freshly isolated strains of streptococci in the green-producing group, I have noted many examples of extreme specificity. Strains culturally and morphologically much alike often manifested widely different localizing and agglutinating properties. Moreover, marked changes in these properties occurred following cultivation on artificial mediums and many successive animal passages.¹ These changes were sometimes so striking as to suggest that a single strain may cause different diseases, depending on peculiar acquired infecting power and other properties, thus supporting clinical and epidemiologic observations. During a mild outbreak of scarlet fever in Rochester in 1917, I noted at the time of infection in one family, a sequence of events which indicated that similar changes occur in the group of hemolytic streptococci. The two members of the family who first became ill developed hemolytic streptococcus infection of the throat, without rash; those next affected developed typical scarlet fever, while those affected last developed only the throat infection.

Epidemics of scarlet fever rarely occur except when hemolytic streptococcus infections of tonsils and throat are prevalent. These and other observations impelled me to immunize two horses at the time of the epidemic, one with hemolytic streptococci from scarlet fever, the other with hemolytic streptococci from septic infection. The epidemic of scarlet fever had disappeared before immunization was complete; hence the serum of the horse immunized with the scarlatinal strains was not used in treatment. The serum from the other horse was used in the treatment of patients with septic infection and erysipelas, with apparent benefit in some instances. Both serums were found to have marked agglutinating power over the strains used for immunization.

Through the researches of G. F. and Gladys H. Dick in scarlet fever,² a new method for the study of hemolytic streptococci has been made

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¹ Jour. Am. Med. Assn., 1915, 65, pp. 1687-1691. Jour. Am. Med. Assn., 1924, 82, pp. 449-453. Jour. Am. Med. Assn., 1913, 60, pp. 1947-1950. Jour. Infect. Dis., 1915, 17, pp. 403-408.

² Jour. Am. Med. Assn., 1923, 81, p. 1166; 1924, 82, p. 265.

available, namely, observation of the toxic action of filtrates and their neutralization by immune serums; through the work of Schultz and Charlton,³ and Main,⁴ the blanching phenomenon with convalescent serum was made available, and through the work of Dochez,⁵ the blanching of scarlatinal rash by experimentally produced immune horse serum.

Experiments on toxicity and neutralization were undertaken, because if serum preserved for 6 years should prove to have neutralizing power over the scarlatinal toxin, it would answer the practical question as to the duration of potency of immune scarlatinal serums, and would indicate that injection of the dead and living streptococci without the broth suffices to produce a neutralizing serum, a point now under discussion. Moreover, it was thought that such experiments, especially with filtrates of hemolytic streptococci from sources other than scarlet fever, might yield greatly needed information with regard to the origin of the scarlatinal hemolytic streptococcus, since it has been shown by G. F. and Gladys H. Dick to be not merely a secondary invader, but the actual cause of scarlet fever. I wish now to report the results obtained in the investigations conducted along these lines.

The scarlatinal serum used was prepared in the horse (horse 10) by repeated inoculation of hemolytic streptococci freshly isolated from the acutely inflamed throats of 3 patients with typical attacks of scarlet fever. The injections were made intravenously on 3 successive days each week from Dec. 19, 1917, to April 20, 1918. The initial injection consisted of the killed bacteria (heated to 56 C. for 45 minutes) from 100 c. c. of 0.2% glucose broth, suspended in 10 c. c. of salt solution. The heat-killed organisms were injected during the first 5 weeks, and live bacteria suspended in salt solution during the remainder of the period of immunization. The dose was gradually increased until the organisms from 600 c. c. of broth were given at a single injection. The horse remained well until the latter part of March, when it developed arthritis, which gradually increased until May 6, when it was bled to death under ether anesthesia. The serum used was obtained from this bleeding and from bleedings made April 8 and May 1. After the serum had been proved sterile, 0.2% tricresol was added, and the serum placed in amber bottles securely stoppered, and kept continuously in the ice chest.

³ Ztschr. f. Kinderh., 1918, 17, p. 328.

⁴ Lancet, 1923, 2, p. 1390.

⁵ Proc. Soc. Exper. Biol. & Med., 1924, 21, p. 184.

Another horse (horse 19), whose serum was used as a control, was immunized in a similar manner with hemolytic streptococci from septic infection.

PRECIPITIN EXPERIMENTS

The precipitin tests consisted of layering various cleared extracts of the streptococci over the respective serums in small glass tubes (3.5 cm. long by 3.5 mm. inside diameter), and noting the presence or absence of a cloud at the juncture of extract and serum after 2 hours' incubation and after being kept in the ice chest over night (fig. 1). The readings were made in a darkened room by transmitted light obtained from below the shade of an electric light bulb, directing the eye into a dark background. In order to avoid undue admixture of extract and serum at the layer of contact, the fluids are added with a pipet drawn to a capillary end. The extract is added first and then the serum. The

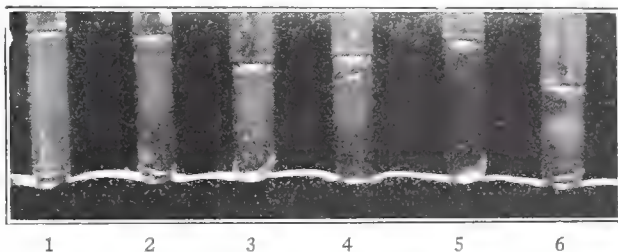


Fig. 1.—Precipitin reaction with various serums, and pooled nasopharyngeal swabbings from patients with scarlet fever. Tubes 1 to 5, negative; tube 6, positive: tube 1, "antipoliomyelitis," serum horse 21; tube 2, "antipoliomyelitis," serum horse 26; tube 3, "anti-encephalitis," serum 89-4; tube 4, antihemolytic streptococcus serum (septic infection) horse 19; tube 5; normal horse serum; tube 6, antihemolytic streptococcus serum (scarlet fever) horse 10.

pipet in both instances is plunged to the bottom of the tube and a column of each, about 1 cm. in height, is allowed to flow in slowly.

In my work on specificity in the group of green-producing streptococci, it was found that specific agglutinating and precipitating properties were maintained over long periods when freshly isolated strains were kept in dense suspension in glycerol (2 parts) and 25% salt solution (1 part). The glycerol-salt solution extracts used in the experiment were made by diluting such dense suspensions of hemolytic streptococci with water to the density of the original broth culture and centrifugating until water-clear. Extracts from old blood-agar slant cultures were made by adding 2 c.c. of distilled water, slanting the tubes so that the water covered the slant, and leaving the tubes over night in the ice chest. The extracts from the throats were made by swabbing the nasopharynx,

washing the swab in 2 c.c. of salt solution, squeezing out the fluid from the cotton, and centrifugating until water-clear. Extracts from old blood-agar slant cultures were made by adding 2 c.c. of distilled water, slanting the tubes so that the water covered the slant, and leaving the tubes over night in the ice chest. The extracts from the throats were made by swabbing the nasopharynx, washing the swab in 2 c.c. of salt solution, squeezing out the fluid from the cotton, and centrifugating until water-clear.

The results obtained with extracts of hemolytic streptococci from scarlet fever and other sources, and with extracts of nasopharyngeal swabbings in cases of scarlet fever and in normal controls, are summarized in table 1. Only negative results were obtained with immune

TABLE 1
PRECIPITIN REACTIONS WITH ANTIGENS FROM HEMOLYTIC STREPTOCOCCI AND FROM SCARLET FEVER

Antigens	Source of Hemolytic Streptococcus	Strains Tested	Positive Reactions with		
			Serum Horse 10 Scarlet Fever	Serum Horse 19 Septic Infection	Serum Normal Horse Control
Cleared glycerol salt solution extract	Scarlet fever	18	17	0	0
Cleared glucose-broth culture	Scarlet fever	12	9	0	0
Cleared water extract of old blood-agar slants	Scarlet fever	5	5	1	0
Cleared glycerol salt solution extract	Miscellaneous	12	2	4	0
Cleared salt solution suspension of nasopharyngeal swabbings from scarlet fever	Scarlet fever	32	17	1	0
Cleared salt solution suspension of nasopharyngeal swabbings from normal persons not exposed to scarlet fever	Normal throat	76	3	0	0

serums used as controls, which were prepared by injecting green-producing streptococci isolated, respectively, in cases of poliomyelitis, encephalitis, chorea and influenza, and with antipneumococcus serums.

The results of the precipitin reaction are in agreement with my own studies in agglutination and with those of Tunncliffe,⁶ Bliss,⁷ Dochez⁵ and others who worked on agglutination reactions, in that they show that the hemolytic streptococci from scarlet fever are quite homogeneous, and that hemolytic streptococci from other sources are more heterogeneous. Nearly all in the scarlet fever group reacted positively with the immune scarlet fever serum, while only 4 of the 12 strains from other sources gave a positive test with the immune serums prepared with

⁶ Jour. Am. Med. Assn., 1920, 74, p. 1386.

⁷ Bull. Johns Hopkins Hosp., 1920, 31, p. 173.

the hemolytic streptococcus from septic infection. The hemolytic streptococcus was demonstrated in the nasopharyngeal swabbings from all but one of the 17 cases of scarlet fever that yielded a positive precipitin test, whereas of the 15 that reacted negatively, it was found in only 3. All tests were positive in the acute stage of the disease.

Since scarlet fever serum has the power to produce a blanching of the rash, it was decided to test its precipitating power. Using normal human serum as a control, the pooled convalescent serum from 3 patients with scarlet fever was tested for the precipitin reaction with nasopharyngeal swabbings from 35 patients with scarlet fever. Of these, 8 gave a mildly positive reaction with the scarlet fever serum. Of 8 patients with poliomyelitis, 1 gave a mildly positive test; of 16 poliomyelitis contacts, two reacted positively, while of 79 normal controls, only 3 gave mildly positive reactions. Blood-agar platings were satisfactory in 11 of the 14 nasopharyngeal swabbings that reacted positively, and all of these showed hemolytic streptococci. The normal serum controls were all negative.

The reaction appears to be specific, and should prove useful in classifying hemolytic streptococci and in differentiating scarlatinal and nonscarlatinal throat infections, and hence should be of epidemiologic and diagnostic value in scarlet fever.

NEUTRALIZATION EXPERIMENTS

The technic of the neutralization experiments was similar to that developed by Dick. The organisms were grown chiefly in human blood broth for from 4 to 7 days, and the cleared supernatant broth culture was passed through bacterial filters of the Mandler type. The neutralizing power of the immune scarlatinal horse serum, convalescent scarlet fever serum and control serums was first tested by mixing equal parts of the serum with the filtrate or dilutions thereof, incubating for 1 hour and injecting 0.2 c c. into the skin.

Severe initial reactions, due to the toxicity of the filtrates; delayed urticarial reactions, due to the protein of the horse serum; the danger of rendering persons sensitive to horse serum and the extreme variations in susceptibility of human beings emphasized the importance of finding a suitable test animal to determine the neutralizing power of this and the newly developed therapeutic scarlatinal horse serums. Many animals were tried. The horse, cow, goat, dog, monkey (*Macacrus rhesus*), rabbit, guinea-pig, white rat, mouse and fowl were all found insusceptible

to intracutaneous injection. The skin over the abdomen of pigs (Chester white) weighing from 20 to 50 pounds (fig. 2) and the skin in the groin and axilla of the sheep and lamb were susceptible.

The reaction in these animals at the site of injection consists of redness and infiltration of varying degree, resembling closely that noted in human beings, but reaches its height in about 8 hours instead of in 24, as in man. The speedier reaction in these animals is in keeping with their higher normal temperature, which averages 3 to 4 degrees higher than that of man. Secondary reactions some days later following injection of mixtures containing horse serum and uninoculated blood-

TABLE 2
TOXICITY OF FILTRATES OF HEMOLYTIC STREPTOCOCCI ON INTRACUTANEOUS INJECTION INTO MAN, PIG AND SHEEP

Hemolytic Streptococcus				Intracutaneous Reaction (June 6, 1924)							
Source	Date Isolated	Strain Number	Filtrate Number	Case 1	Case 2	Pig 1	Pig 6	Sheep 3	Sheep 4	Sheep 2	
				1:100 1:1000	1:100 1:1000	1:1 1:10 1:100 1:1000	1:1 1:10 1:100 1:1000	1:1 1:10 1:100 1:1000	1:1 1:10 1:100 1:1000	1:1	
Scarlet fever	March 2, 1918	1080.6	1	0 0	1 2	1 0 0 0	1 0 0 0	0 0 0 0	0 0 0 0	0	
	May 8, 1920	3916.12	2	4 3	3 2	3 2 1 0	3 2 1 0	2 0 0 0	3 0 0 0	3	
	May 12, 1924	22.5	5	3 2	3 2	3 2 1 0	3 2 1 0	3 1 0 0	4 3 2 0	4	
	May 12, 1924	23.5	6	3 2	2 2	4 3 1 0	3 2 1 0	3 1 0 0	4 3 2 1	3	
	May 12, 1924	24.5	7	4 3	3 3	3 2 0 0	2 1 0 0	2 1 0 0	4 4 3 2	3	
Septic infection	March 2, 1918	1081.6	14	3 2	2 2	3 3 2 1	4 2 2 0	2 0 0 0	4 3 2 0	1	
	April 16, 1918	2037.8	19	3 1	3 2	1 1 0 0	1 1 0 0	2 0 0 0	3 2 2 0	1	
Filtrate of uninoculated blood broth...				0	0	0	0	0	0	0	
Sodium chloride solution.....				0	0	0	0	0	0	0	

broth filtrates, and pseudo reactions rarely occurred. Generalized reactions with fever were not observed, and the reactivity was not perceptibly altered after repeated intradermal injections.

Parallel toxicity and neutralization experiments in man, pig and sheep, gave virtually the same results (table 2), although the pig was found most uniformly satisfactory. The susceptibility of the pig and sheep is approximately one-tenth as great as that of man. The best results were obtained in these species when filtrates were diluted from 1:10 to 1:100, whereas dilutions of from 1:100 to 1:1,000 were found best suited to man. Reactions at the site of injection at the end of 24 hours in man, and after 8 hours in the pig and sheep, were recorded in the tables on a basis of 1:4, as follows: Slight redness and induration,



Fig. 2.—Neutralization in the pig of the toxin of a hemolytic streptococcus recently isolated in scarlet fever, by the immune scarlatinal serum, horse 10, prepared 6½ years previously. Dilutions: 1:1, 1:10, 1:100, 1:1,000; upper row, without serum; lower row, with serum (see result in pig 2, table 7).

1 or (+); moderate redness and induration, about 1 cm. in diameter, 2 or (++) ; marked reaction from 1 to 2 cm. in diameter, 3 or (+++), and still greater reactions, 4 or (++++) . The toxicity of filtrates varied greatly according to the nature of the culture medium and the quality of the strains. Blood-broth cultures from 4 to 7 days old yielded filtrates of highest and most uniform toxicity.

The results recorded in table 3. show the toxicity of filtrates of hemolytic streptococci isolated in scarlet fever and other diseases, as

TABLE 3
TOXICITY OF FILTRATES OF BLOOD-BROTH CULTURES OF HEMOLYTIC STREPTOCOCCI, ON
INTRACUTANEOUS INJECTION INTO PIGS

Hemolytic Streptococcus				Intracutaneous Reaction (June, 1924)			
Source	Strain	Date Isolated	Filtrate	Pig 3	Pig 4	Pig 5	Pig 7
Scarlet fever	1080.6	March 2, 1918.....	1	++	+	+++	+
	3916.12	May 8, 1920.....	2	+++	++	++++	+++
			2 a*	+	+	+	6
	3922.15	May 12, 1920.....	3	+	+	++	+
	4128.16	Jan. 22, 1921.....	4	++	—	+	+
	22.5	May 12, 1924.....	5	++	—	++++	++++
	23.5	May 12, 1924.....	6	+++	+++	+++	+++
			6 a*	+	+	+	0
	24.5	May 12, 1924.....	7	+++	+++	+++	++
	25.5	May 12, 1924.....	8	++	++	++	+
	26.5	May 12, 1924.....	9	++	++	++	+
	27.5	May 12, 1924.....	10	++	+++	++	++
	28.5	May 12, 1924.....	11	+++	+++	++++	++
Other diseases	29.5	May 12, 1924.....	12	+	+	+++	++
	31.5	May 12, 1924.....	13	++	++	++	+++
	1081	March 2, 1918.....	14	+++	+++	++++	++++
	2012 ² .12	April 8, 1918.....	15	+	++	+	+++
	2013	April 8, 1918.....	16	+	++	+	+
	2032.13	April 13, 1918.....	17	+	++	+	++
	2036.13	April 15, 1918.....	18	+	++	+	++
	2037.8	April 16, 1918.....	19	++	++	+++	+++
	2697	Dec. 14, 1918.....	20	++	++	++	++
	3077.7	Feb. 25, 1919.....	21	+++	++	++++	+++
	3250.18	March 22, 1919.....	22	+	++	++	++
	3673.5	Dec. 5, 1919.....	23	++	++	+++	++++
	2577.12	About 40 years ago.....	24	+	+	+++	++
			24 a*	+	+	+	+

* From blood broth to which fresh sterile kidney was added at time of inoculation.

measured on intracutaneous injection into the pig. All of the 24 filtrates were obtained from 4-day cultures of the respective strains in meat-infusion broth containing 1 part of human defibrinated blood to 8 parts of broth. The blood obtained from 6 persons was previously mixed, and the broth was all from one batch. The total quantity of blood broth used for each strain was 60 c.c., contained in bottles making a column 6 cm. tall. Blood-agar platings were made the day before filtration to determine the amount of growth. Bacterial filters of the Mandler type were used, and all filtrates were proved sterile before injection. No preservative was added.

Fifteen of the 24 strains tested in the experiment recorded in table 3, yielded filtrates of high toxicity, while 9 were of low toxicity. Of the 13 strains isolated from patients with scarlet fever, 9 yielded highly toxic filtrates, while 4 had slight toxicity. Two of 4 scarlet fever strains isolated long before produced highly toxic filtrates. One of these was isolated 6 years, the other 4 years, previously. Seven of 9 freshly

TABLE 4
NEUTRALIZING POWER OF SCARLATINAL ANTISTREPTOCOCCUS HORSE SERUM OVER FILTRATES OF STREPTOCOCCI ON INTRACUTANEOUS INJECTION INTO MAN AND PIG

Filtrate				Intracutaneous Reaction				
Number	Streptococcus		Treated with Equal Volume of	Dilutions: 1:1 1:10 1:100 1:1000				
	Type	Source						
23	Hemo-lytic	Scarlet fever	Salt solution	Case 1	++++	+++	+++	++
				Case 2	++++	++++	++++	++++
				Pig 1	++++	+++	++	++
				Pig 2	++++	+++	+++	++
			Scarlatinal antistreptococcus serum horse 10	Case 1	++	+	0	0
				Case 2	+++	++	+	0
				Pig 1	+++	+++	+	0
				Pig 2	+++	+++	0	0
			Antistreptococcus (hemolytic) serum of septic infection horse 19	Case 1	+++	++	+	+
				Case 2	+++	+++	++	++
				Pig 1	++++	+++	++	+
				Pig 2	+++	++	0	0
Mixture of ten filtrates	Hemo-lytic	Scarlet fever	Salt solution	Case 1	+++	+++	++	++
				Case 2	++++	+++	+++	+++
				Pig 1	+++	+++	++	+
				Pig 2	++	++	+	+
			Scarlatinal antistreptococcus serum horse 10	Case 1	+	0	0	0
				Case 2	++	+	0	0
				Pig 1	++	++	0	0
				Pig 2	0	0	0	0
			Salt solution	Case 1	+	+	0	0
				Case 2	0	0	0	0
				Pig 1	+	0	0	0
				Pig 2	0	0	0	0
257	Hemo-lytic	Septic infection	Salt solution	Case 1	+	+	0	0
				Case 2	0	0	0	0
				Pig 1	+	0	0	0
				Pig 2	0	0	0	0
35	Viridans	Chorea	Salt solution	Case 1	+	0	0	0
				Case 2	0	0	0	0
				Pig 1	0	0	0	0
				Pig 2	0	0	0	0

isolated scarlet fever strains produced filtrates of high toxicity. Three freshly isolated strains of hemolytic streptococci from postscarlatinal otitis media, which occurred one month after typical attacks of scarlet fever, produced highly toxic filtrates. Six of the strains isolated long before from nonscarlatinal sources yielded highly toxic filtrates, and 5 yielded filtrates slightly toxic. The addition of fresh sterile kidney to the blood broth just before inoculating interfered markedly with the production of toxin.

The hemolytic streptococci from nonscarlatinal sources included strains from cases of ascending lymphangitis, acute follicular tonsillitis, otitis media, mastoiditis, erysipelas, pneumonia, empyema, peritonitis, and acute cholecystitis. One strain was isolated from cheese during the epidemic of empyema and pneumonia at Fort Riley in 1918. In no instance were these infections associated with scarlet fever.

Table 4 shows slight neutralizing power of the nonscarlatinal hemolytic streptococcus serum (horse 19) and marked neutralizing power of the antiscarlatinal serum (horse 10) over the toxin contained in the filtrate of a recently isolated scarlatinal strain and that contained in a mixture of filtrates from 10 scarlatinal strains. The results are similar in man and in the pig. The filtrates of the hemolytic streptococcus (257) from septic infection, isolated by Professor Holst of the University of Christiania over 40 years before, and that of the green-producing streptococcus (35) from chorea, injected as controls, had little or no toxic action. Experiments in which progressive dilutions of the immune scarlatinal serum were used showed that it had measurable neutralizing power in dilutions as high as 1:200 in salt solution. The cutaneous reactions of filtrates from scarlatinal and from nonscarlatinal strains were identical.

Parallel neutralization experiments were next made with filtrates of scarlatinal and of nonscarlatinal strains. The scarlatinal immune serum neutralized the filtrates from nonscarlatinal strains as markedly as those from scarlatinal strains. This was also true in repeated experiments with the corresponding antibody solution prepared by the dilution method used by Felton in obtaining pneumococcus antibody solution. The results of tests with controls, serums and antibody solutions on each of these types of filtrates are summarized in table 5.

Marked neutralization of the toxicity of both types of filtrates for man, pig and sheep followed treatment with the scarlatinal immune serum and corresponding antibody solution; slight reduction in toxicity followed treatment with the nonscarlatinal immune serum and antibody solution, while no diminution of toxicity resulted from treatment with antistreptococcus (chorea) serum and antibody solution and with normal horse serum (table 5).

If the toxic elements in these filtrates of scarlatinal and nonscarlatinal strains are really identical, then convalescent scarlet fever serum and the serum from persons immunized with scarlatinal toxin should neutralize both sets of filtrates. This has been found to be the case in repeated

tests. The immune human serums were obtained 10 days after severe reactions to intracutaneous injection of scarlatinal toxins, and the convalescent serums 7 days (case 25) and 14 days (case 26) after onset of the attack of scarlet fever. The results of one such experiment are given in table 6.

TABLE 5
NEUTRALIZATION BY SCARLATINAL ANTISTREPTOCOCCUS HORSE SERUM OF TOXIN IN FILTRATES OF HEMOLYTIC STREPTOCOCCI FROM SCARLET FEVER AND FROM SEPTIC INFECTION

Filtrate (Diluted 1:5 with Salt Solution) Treated with Equal Parts of	Intracutaneous Reaction											
	Filtrate 5 (Scarlet Fever)						Filtrate 14 (Septic Infection)					
	Case 5	Case 4	Pig 1	Pig 2	Sheep 3	Sheep 4	Case 3	Case 4	Pig 1	Pig 2	Sheep 3	Sheep 4
Salt solution.....	++	++	++	+++	++	++	+	++	++	+++	++	++
Antistreptococcus (scarlet fever) serum, horse 10.....	0	0	+	+	0	+	0	0	+	+	0	+
Pseudoglobulin anti- body solution serum, horse 10.....	0	+	0	+	+	9	0	+	+	++	+	+
Antistreptococcus (septic infection) serum, horse 19.....	+	+	++	++	++	++	+	++	++	+++	++	++
Pseudoglobulin anti- body solution serum, horse 19.....	++	+	+	+++	++	++	+	+	++	++	++	++
Antistreptococcus (chorea) serum, horse 25.....	++	++	++	+++	++	++	+	++	++	+++	++	++
Pseudoglobulin anti- body solution serum, horse 25.....	++	++	++	+++	++	++	+	++	++	++	++	++
Normal horse serum....	++	++	++	+++	++	++	+	++	++	+++	++	++

TABLE 6
NEUTRALIZATION BY CONVALESCENT SCARLET FEVER SERUM OF THE TOXIN IN FILTRATES OF HEMOLYTIC STREPTOCOCCI FROM SCARLET FEVER AND FROM SEPTIC INFECTION

Filtrate (Diluted 1:5 with Salt Solution) Treated with Equal Parts of	Intracutaneous Reaction					
	Filtrate 5 (Scarlet Fever)			Filtrate 14 (Septic Infection)		
	Pig 8	Pig 9	Lamb 4	Pig 8	Pig 9	Lamb 4
Salt solution.....	+++	+++	+++	+++	+++	+++
Normal human serum.....	+++	+++	++	+++	+++	+++
Immune human serum (Cases 1 and 2)	0	0	0	0	0	9
Immune human serum (Cases 3 and 4)	++	+	0	+	+	++
Convalescent scarlet fever serum (Case 25).....	+	+	0	+	+	+
Convalescent scarlet fever serum (Case 26).....	0	0	++	0	9	+

Moreover, if the two types of hemolytic streptococci are identical, both types or organisms should absorb the "antitoxin" contained in the neutralizing scarlatinal serum and other streptococci should not. Absorption experiments were therefore made. One part of a dense salt solution suspension (50 times the density of the dextrose-broth cultures) of the



Fig. 3.—Absorption of antitoxin from scarlatinal serum of horse 10, with scarlatinal and nonscarlatinal hemolytic streptococci. Top row: Scarlatinal and nonscarlatinal toxins plus untreated serum; second row: toxins plus serum previously treated with scarlatinal hemolytic streptococci; third row: toxins plus serum previously treated with nonscarlatinal hemolytic streptococci; fourth row: toxins plus serum previously treated with green-producing streptococci from poliomyelitis.

washed and heat-killed organisms (heated to 60 C. for 45 minutes) was mixed with 9 parts of serum, corresponding antibody solution or salt solution, incubated for 1 hour and centrifugated to water clearness. This was repeated once, incubating the mixtures for 1 hour, then placing them in the refrigerator over night and again centrifugating. The serum and antibody solution caused agglutination of both types of hemolytic streptococci, but not of the poliomyelitis strains. The results recorded in table 7 show that the antitoxin in the scarlatinal immune serum and antibody solution was neutralized or absorbed by the scarlatinal and non-

TABLE 7

ABSORPTION OF THE NEUTRALIZING PRINCIPLE IN SCARLATINAL IMMUNE SERUM AND ANTIBODY SOLUTION BY SCARLATINAL AND NONSCARLATINAL HEMOLYTIC STREPTOCOCCI

Serum	Treated with	Intracutaneous Reaction, Diluted Filtrate (1:250) Treated with Equal Parts of Serum			
		Filtrate 22.7 (Scarlet Fever)		Filtrate 1081 (Septic Infection)	
		Case 1	Case 2	Case 1	Case 2
Serum, horse 10.....	Scarlatinal streptococci (strain 22.7).....	0	0	0	0
	Nonscarlatinal hemolytic streptococci (strain 1081)...	++++	++++	++++	+++
	Green-producing streptococci from poliomyelitis...	++++	+++	++++	+++
		0	0	0	0
Antibody solution serum, horse 10.....	Scarlatinal streptococci.....	0	0	0	0
	Nonscarlatinal hemolytic streptococci.....	++++	+++	++++	++
	Green-producing streptococci from poliomyelitis...	++++	++++	+++	++++
		+	+	+	++
Salt solution.....	Scarlatinal streptococci.....	++++	+++	+++	+++
	Nonscarlatinal hemolytic streptococci.....	+++	+++	++++	+++
	Green-producing streptococci from poliomyelitis...	+++	+++	+++	+++
		+++	++	+++	++

scarlatinal hemolytic streptococci, but not by the control poliomyelitis streptococci. The results of the first 4 tests in case 2 are well illustrated in figure 3.

Since I have shown in epidemic hiccup and ulcer of the stomach that specific localization and lesions followed injection of the respective living cultures, dead bacteria and filtrates, it was assumed that the characteristic toxin would also be present in hemolytic streptococci. If this were so, then the washed and heat-killed organisms, after treatment with the immune serum and antibody solution, should cause less local reaction on intracutaneous injection than the untreated organisms suspended in equivalent amounts of salt solution. The results obtained in two persons receiving parallel injections proved this assumption to be correct.

It was not possible adequately to test the value of this old scarlatinal horse serum in the treatment of cases of scarlet fever, owing to the small quantity available; however, it was found to cause distinct blanching of the rash in several cases of scarlet fever.

The experiments on toxicity and neutralization referred to were made during June, 1924. The filtrates were then placed in the refrigerator and kept until December. A new series of experiments was undertaken to test the keeping qualities of the toxin and the reactivity of the pigs, which had grown until they weighed approximately 100 pounds, also to repeat similar experiments with newly prepared toxins, and to repeat the experiments on toxicity in the rabbit, because of the positive results in the animal reported by Williams, Hussey and Banzhof. All of the old filtrates, including one filtrate sent me last August by Dr. Dick, had lost most of their toxicity. The antiscarlatinal serum (horse 10) and a newly prepared antibody solution again neutralized freshly prepared filtrates from scarlatinal and nonscarlatinal strains in man and in the hog. The results in the hog were as satisfactory as in the pig. Neutralization of toxins diluted as low as 1:20 occurred with dilutions of serum as high as 1:200. Further tests in the rabbit again proved negative; rabbits of different color, breed and age received injections of undiluted and diluted filtrates, all with entirely negative results.

SUMMARY

The experiments on toxicity and neutralization not only support the newer results obtained in scarlet fever by G. F. and Gladys H. Dick and by Dochez, but also show that great differences occur in the toxin-producing power of scarlatinal and nonscarlatinal strains of hemolytic streptococci, and that certain strains possess this power to a high degree years after isolation. Moreover, they show that it is possible to produce in the horse a neutralizing serum by intravenous injection of dead and living hemolytic streptococci from scarlet fever, a result within expectations because of the demonstrated identity of the toxin in filtrates and that within the washed dead organisms. On the basis of Dochez's results, it may be that our efforts were successful, because the horse developed arthritis.

In the pig or hog (Chester white) was found what promises to be a suitable animal for determining the toxicity of filtrates from cultures of hemolytic streptococci and perhaps for the standardization of antiscarlatinal serums. Good keeping qualities of antiscarlatinal serums

may confidently be expected, since specific precipitating and neutralizing properties were demonstrated in the serum prepared more than 6 years before.

The large amount of toxin produced by the 3 strains of hemolytic streptococci isolated in cases of postscarlatinal otitis media, which was neutralized by the scarlatinal serum, indicates that these strains would have produced scarlet fever in susceptible persons, and that immunity of the skin to the toxin does not necessarily mean immunity of the throat to infection by truly scarlatinal hemolytic streptococci.

Preservation of filtrates in the ice chest for 6 months markedly reduced their toxicity.

Identity of the soluble toxin produced by scarlatinal and certain non-scarlatinal strains seems certain, since both were neutralized by the immune scarlatinal horse serum, by the serum from persons immunized with scarlatinal filtrates (the Dick method) and by convalescent scarlet fever serum. The fact that the neutralizing principle (antitoxin) was removed from the scarlatinal serum by the washed bacterial bodies of both types of hemolytic streptococci, and that the "endotoxin" within the heat-killed bacterial cells was neutralized equally in both, is further proof of the identity of the soluble toxin and endotoxin of scarlatinal and of certain nonscarlatinal strains.

On the basis of these results, may not specificity in the scarlatinal hemolytic streptococcus be an expression of high toxin-producing power and of other peculiar acquired properties in the hemolytic streptococcus group?

METHYLENE VIOLET AND METHYLENE AZURE A AND B*

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In 1906, in this Journal,¹ there was presented a review of the chemistry of the polychrome methylene blue dyes and of their application as stains in pathology. A practical method for the preparation of methylene violet free from methylene blue and methylene azure and an easy method of preparing methylene azure free from methylene violet and methylene blue were described in that earlier publication. Since 1906, we have continuously employed in practical staining of blood films, staining solutions made by mixing together the 4 essential dyes: eosin, methylene blue, methylene violet and methylene azure. Descriptions of the stains employed have been published from time to time.² The renewed interest in this field indicated by the discussion between Unna³ and Giemsa⁴ in Germany, the publications of Scott and French⁵ and of Proescher and Krueger⁶ in this country, and the evident intent of American chemists to carry on the manufacture of these dyes may be regarded as an adequate excuse for presenting at this time some further observations made during the last 18 years, together with a consideration of the more important facts revealed in the literature during this time.

Kehrmann,⁷ in 1906, undertook the study of methylene azure in order to defend his conception of the thionium structure of methylene blue as against the parachinon linking given by Bernthsen.⁸ In this

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¹ Mac Neal, Ward J.: Jour. Infect. Dis., 1906, 3, p. 412.

² Mac Neal, Ward J.: Jour. Am. Med. Assn., 1907, 48, 609. Mac Neal, Ward J., and Schule, Paul A.: The Post-Graduate, 1913, 28, p. 982. Mac Neal, Ward J.: Pathogenic Microorganisms, Blakiston, Philadelphia, 1920, p. 44; Tetrachrome Blood Stain: an Economical and Satisfactory Imitation of Leishman's Stain, Proc. N. Y. Pathol. Soc., 1922, 22, p. 41; Jour. Am. Med. Assn., 1922, 78, p. 1122.

³ Centralbl. f. Bakteriologie, I. O., 1922, 88, p. 159. Baudisch, O., and Unna, P. G.: Dermat. Wehnschr., 1919, 68, p. 49.

⁴ Centralbl. f. Bakteriologie, I. O., 1924, 91, p. 343. Giemsa, G.: Ibid., 1922, 88, p. 99.

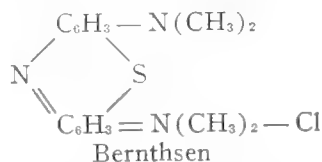
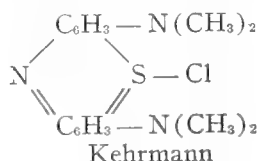
⁵ Mil. Surgeon, Aug. and Sept., 1924.

⁶ Jour. Lab. & Clin. Med., 1924, 10, p. 153.

⁷ Ber. d. deutsch. chem. Gesellsch., 1906, 39, p. 1403. Kehrmann, F.; Havas, E., and Grandmougin, E.: Ibid., 1913, 46, p. 2131.

⁸ Ann. d. Chem., 1885, 230, p. 73; Ber. d. deutsch. chem. Gesellsch., 1906, 39, p. 1804.

work, he received the hearty cooperation of Bernthsen. As a result of the study, Kehrman's orthochinon formula has been accepted as a



possible structural picture of the methylene blue molecule and is often given the preference in modern publications. This theoretical phase we shall reserve for later discussion, but it may be said that our own work appears to favor the older structural formula of Bernthsen.

The important practical achievement of Kehrman was the separation from polychrome methylene blue of trimethyl thionin and the asymmetric dimethyl thionin. He was also able to prepare the latter synthetically by action of dimethylamine on mono-amino-phenazthionium chloride, thus proving the structural formula. This substance he designated as methylene azure A. The trimethyl thionin he called methylene azure B. Kehrman also prepared the symmetrical dimethyl thionin, but he found nothing corresponding to it in polychrome methylene blue. Among the disintegration products of the action of alkali on methylene blue, he recognized leukomethylene blue, methylene violet, methylene azure A (dimethyl thionin) and methylene azure B (trimethyl thionin).

Scott and French⁵ have evidently followed the method of Kehrman, namely, oxidation of methylene blue in alkaline solution at low temperature in the preparation of azure. It would appear, however, that they have not employed the older chemical tests in order to recognize the nature of the substances with which they are dealing, but have placed chief reliance on precise determination of physical color values by spectrometric studies. Important as the latter may be, they cannot replace such criteria as crystal form, color changes and solubility in water, chloroform and ether as influenced by pure reagents, such as sulphuric acid, hydrochloric acid, sodium chloride, sodium hydroxide, acetic acid and sodium bicarbonate.

More recently Proescher and Krueger⁶ have suggested preparing polychrome methylene blue by the action of sodium peroxide, Na_2O_2 , on methylene blue, and Marie and Raleigh⁹ have made polychrome

⁹ Jour. Lab. & Clin. Med., 1924, 10, p. 250.

methylene blue by the action of ultraviolet light on alkaline methylene blue solution. These are obviously empiric methods resulting in mixtures of unknown composition.

DIMETHYL THIONIN (METHYLENE AZURE A)

Dimethyl thionin is easily prepared by oxidizing methylene blue in very dilute acid with potassium chromate or potassium dichromate. This reaction takes place in 3 distinct stages. First, on mixing the dye with

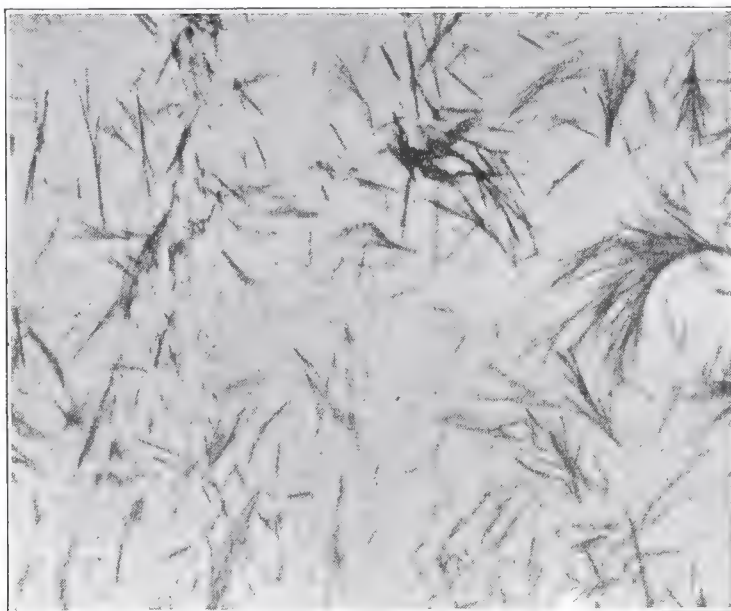
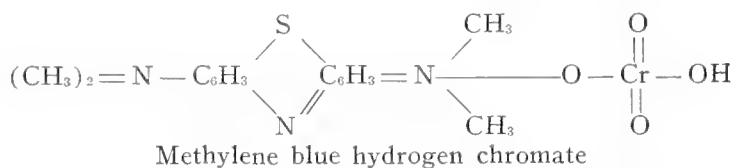


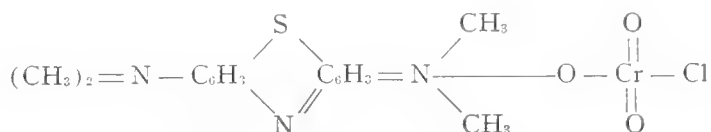
Fig. 1.—Crystals produced by adding dichromate and hydrochloric acid to dilute methylene blue solution.

the chrome salt, there is produced a flocculated amorphous purple precipitate which, without an ultimate analysis, would appear to be either methylene blue hydrogen chromate or dimethylene blue chromate.



On the addition of hydrochloric acid, this purple precipitate changes to steel blue, sharp, stiff needle crystals, slowly at room temperature, but

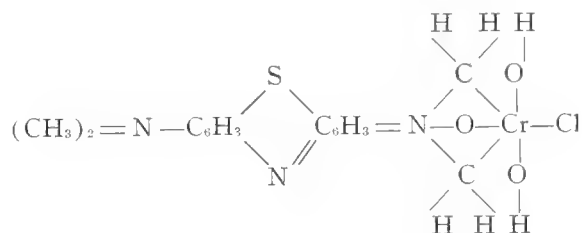
rapidly when hot. This crystalline precipitate is almost insoluble in water. It contains chromium and chlorine and appears to be a chlor-chromate of methylene blue.¹⁰



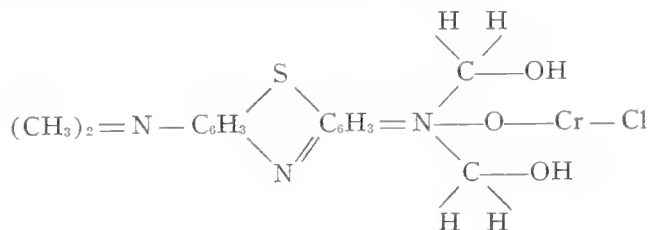
Methylene blue chlor-chromate

When dry it forms a compact mass of bronze copper color.

On heating this substance in dilute hydrochloric acid containing potassium dichromate, it passes slowly into solution, with the production of a deep purple color and the evolution of abundant formaldehyde, the reaction being almost quantitative if not too much acid is used. This reaction is somewhat more complex than the preceding steps, but is obviously an oxidation of the 2 adjacent methyl groups by the oxygen of the chromate radical. The chromium then splits off as Cr Cl_2 to be at once oxidized to Cr Cl_3 and leaves the chloride of dimethyl thionin. The stages of this transformation may be pictured as follows:

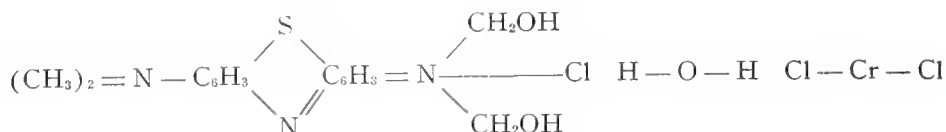


First a transfer of one bond of the chromium to each of the methyl carbon atoms with a transfer of the corresponding hydrogen atom to the oxygen of the chromate; then the complete reduction of the chromate by transfer of the hydroxyl to the methylene group.

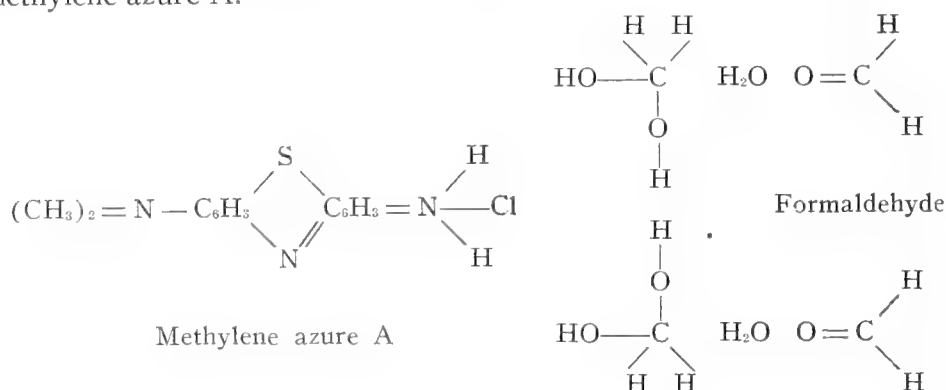


¹⁰ Ultimate analysis of this crystalline substance shows it to be dimethylene blue chromate.

The linking of the chromium to the dye radical next appears to be broken by the interposition of two molecules of hydrochloric acid to form chromous chloride, Cr Cl_2 .



Finally, by a simple hydration, the 2 original methyl groups are eliminated as formaldehyde, leaving dimethyl thionin chloride, or methylene azure A.



The azure dye is readily salted out by saturation with sodium chloride. This precipitate is very soluble in hot alcohol, and crystallizes on cooling as slender, somewhat curved filaments and needles of dimethyl thionin chloride. The substance is characteristic in its chemical reactions and in its staining properties.

In the practical preparation, the quantitative relations of the various substances and the temperature of mixture are important considerations. It is also well to check the form of every precipitate by microscopic examination of it. In general, by using more water, so that the reactions occur in dilute solution, one obtains a purer product. However, the inconvenience of handling large volumes as well as the loss of material remaining in solution has to be considered. Specific directions follow:

Dissolve medicinal methylene blue, 8 gm. ($\frac{1}{40}$ gm. molecule), in 2,000 c.c. water by warming to about 40 C. With constant stirring, add potassium dichromate, 50 c.c. of a 10% solution ($\frac{1}{80}$ gm. molecule is contained in 36.8 c.c.) and hydrochloric acid, 30 c.c. of concentrated (36%) acid in 200 c.c. of water. Bring the mixture to boiling and boil for 1 to 2 hours, until only a faint trace

of methylene blue remains according to the test given in an earlier paper.¹¹ Now pour the hot reaction mixture on to 775 gm. of sodium chloride in a large crystallizing dish, stir thoroughly and let it stand over night. Filter and dry the precipitate between blotters at 37 C. Then dissolve it in successive portions of boiling alcohol (96%), using about 1,000 c.c. in all. Filter these into the same flask. Distil off the alcohol until the volume is reduced to about 400 c.c. Transfer to a beaker, cover and cool slowly to a temperature of 0 C. The material should solidify. By suction filter the liquid is removed from the crystalline precipitate, and the latter is dried at 37 C. between blotters or porous plates. The yield is from 4 to 6 gm. The final product is a feltwork of slender flexible filamentous crystals, green by reflected light.



Fig. 2.—Dimethyl thionin recrystallized from ethyl alcohol.

TRIMETHYL THIONIN (METHYLENE AZURE B)

It has so far been impossible to prepare a satisfactory tri-methyl thionin by the acid-chromate method. By carrying out the reaction in concentrated solution with one-half the amount of chromate and one-fourth the amount of water, one obtains a more soluble crystalline dye substance, which consists of coarser blunt needles. This gives a strong positive test for the presence of methylene blue. Such staining tests as we have made with this substance suggest that it is of no particular

¹¹ Dilute 1 drop of the reaction mixture with 2 c.c. of cold water. Add dilute (decinormal) NaOH until the color changes to purplish red. Add 5 c.c. of ether, shake and allow the ether to separate. A blue tint in the watery portion indicates the presence of methylene blue.

importance as a dye in polychrome methylene blue, as it appears to behave like a mixture of azure A (dimethyl thionin) and methylene blue (tetramethyl thionin). Further study may, however, change our opinion.

METHYLENE VIOLET (BERNTHSEN)

The "Roth aus Methylen-Blau" extracted from alkaline polychrome methylene blue by ether or chloroform is a mixture of methylene azure bases and methylene violet. If the extraction is made with ether in the presence of bicarbonate, the red dye obtained is methylene violet. This



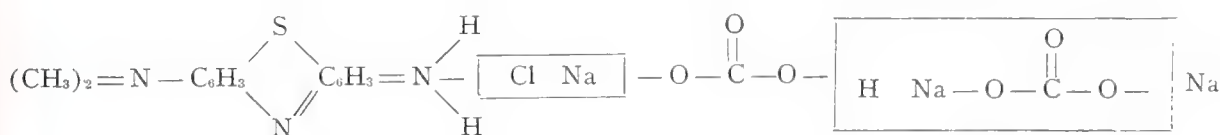
Fig. 3.—Methylene violet recrystallized from ethylene dichloride.

substance has been one of the most elusive of the dyes in this group. It has been difficult to prepare, and its properties have been studied by few investigators. Bernthsen prepared it by shaking a dilute solution of methylene blue iodide with an excess of freshly-prepared silver oxide for a long time, and then boiling the iodine-free filtrate for 12 to 36 hours. The precipitate of methylene violet was recrystallized from alcohol. In our earlier work, methylene violet was prepared by Bernthsen's method, and later by boiling dilute methylene blue to which sodium carbonate had been added. Recrystallization from alcohol has always been very difficult, apparently because other substances are

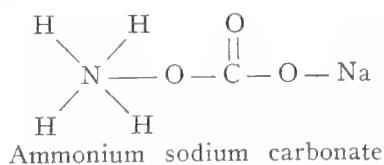
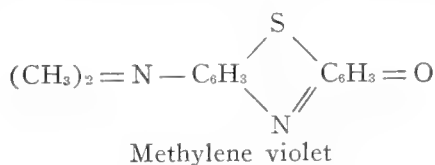
produced by these methods—especially the leukobase of methylene blue—which interfere with crystallization.

Recently it has been found that methylene violet can be more easily made from dimethyl thionin (azure A). One dissolves 4 gm. of azure in 2,000 c.c. of water, adds 2 gm. of baking soda (NaHCO_3) and brings the solution to a boil. While boiling continues, there is added drop by drop 20 c.c. of a 10% solution of sodium carbonate, Na_2CO_3 . Boiling is continued about an hour. Abundant ammonia is evolved. The precipitate is crystalline and consists of methylene violet, pure enough to yield needle crystals with dilute hydrochloric acid. The reaction has to be carried out without making the solution too alkaline. Precipitation of the azure base should be avoided.

The reaction appears to proceed according to the following scheme:

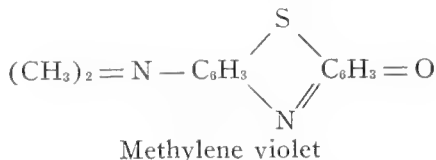


Dimethyl thionin chloride changes to the acid carbonate, neutral carbonate and later to the dimethyl thionin sodium carbonate as the boiling drives off carbon dioxide and the alkaline carbonate is added. Finally, at the proper alkalinity, the double linking of the adjacent nitrogen to the color ring is broken by hydration, setting free $\text{NH}_4-\text{CO}_3-\text{Na}$, from which ammonia is given off in the steam, and precipitating the methylene-violet free base.



Because of the success of this reaction, the preparation of methylene violet from methylene blue by oxidation and hydration in alkaline solution was undertaken. Potassium chromate (yellow chromate) K_2CrO_4 , 48.5 c.c. of 10% solution, is added to a warm (40 C.) solution of methylene blue 8 gm. in 4,000 c.c. of water. After mixing, one adds 15 c.c. of 30% sodium carbonate (Na_2CO_3) and allows the mixture to stand at about 40 C. for an hour or two. It is then brought to a boil and boiled for 3 hours. A small amount of amorphous precipitate appears before boiling begins, but after boiling for 10 minutes, long crystals of methylene violet appear in irregular bundles. Ammonia is evolved in the steam. The precipitate is separated by filtration, washed with water and dried at 37 C. It is then dissolved in successive portions of boiling pure ethylene dichloride (redistilled), these portions being filtered into the same flask. About 1,000 c.c. of the solvent is required. This solution is

then boiled, the solvent being distilled off until the volume is about 300 c.c., poured into a beaker and set aside at 0 C. for 1 to 3 days to crystallize. Beautiful bundles of green shining needles are obtained. The yield, however, is not large, only from 1 to 3 gm. Possibly this may be improved with further experience.



Methylene violet is insoluble in water, but soluble in aqueous methylene blue solution, in ether, chloroform, methyl and ethyl alcohol. It also reacts in a characteristic fashion with dilute hydrochloric acid, forming a violet blue solution when warm, and precipitating as brown needle crystals on cooling. When dissolved in methylene blue solution, it confers on this solution the property of giving the red nuclear color of the Romanowsky stain. The addition of methylene azure, however, improves the stain in this respect.

The tetrachrome stain, which is highly satisfactory for staining blood films, is essentially an imitation of the Leishman stain. It is prepared by mixing definite quantities of its constituents, as follows:

Methylene blue hydrochloride (medicinal U. S. P.)....	1.0
Methylene azure A (dimethyl-thionin chloride).....	0.6
Methylene violet (Bernthsen) free base.....	0.2
Eosin, yellowish, water soluble.....	1.0
Methyl alcohol, absolute, acetone free.....	1000.0

The mixture should be heated to 50 C., thoroughly shaken and left at 37 C. for a day or two, with occasional shaking to complete the solution. This is accomplished more promptly, however, if pure crystalline dyes are employed.

SUMMARY

In polychrome methylene blue, there are at least 3 nuclear dyes which produce a purple color, namely, methylene violet, methylene azure A (asymmetric dimethyl thionin) and methylene azure B (trimethyl thionin).

Simplified and relatively easy methods are described for the preparation of nearly pure crystalline methylene azure A and methylene violet.

By mixing these two substances in definite proportions with methylene blue and eosin and dissolving the mixture in methyl alcohol, one prepares the tetrachrome blood stain, which is highly satisfactory in the staining of blood films.

HEMOLYTIC PROPERTIES OF WHOLE CULTURES AND OF SEDIMENTS OF HEMOLYTIC STREPTOCOCCI

NOBLE P. SHERWOOD AND CORNELIA M. DOWNS

From the Department of Bacteriology, University of Kansas, Lawrence

Bordet¹ observed that in rabbits dying of streptococcus septicemia some lysis in the blood frequently occurred. Besredka² demonstrated the presence of a filtrable hemolytic substance or streptolysin in broth cultures of a certain streptococci. The mechanism of the action of streptolysin was carefully studied by Ruediger,³ who found that serum encouraged its production; that it is inactivated or destroyed at 70 C. for 2 hours; that it gradually deteriorates at room temperature but rapidly at incubator temperature; that it may be preserved for some time in the icebox; that it is destroyed by peptic digestion and is not dialysable. The serum of some animals contain antistreptolysin, but this is not increased by immunization. He concluded that the streptolysin possessed a haptophore and a toxophore group. Since the work of Ruediger, little has been added to our knowledge of the mechanism of the action of streptolysin. Van Hellens⁴ states that streptolysin is heat resistant. Mishulow⁵ noted the sudden disappearance of hemolytic power at icebox temperature. De Kruif and Ireland⁶ have studied the effect of the concentration of various serums on its production, and also the relationship of outpouring of streptolysin with the logarithmic period of growth. In all their work, supernatant fluids obtained by centrifuging cultures at high speed was substituted for filtration. The phenomenon of lytic action of sediments and of whole broth cultures of varying ages was not investigated especially by them, but they assumed it to be due to the same hemotoxin acting at the surface of the organism.

Our object was to study the phenomena of lysis by sediments and whole broth cultures of hemolytic streptococci using plain broth and serum broth of different ages and comparing the results, using varying amounts of red blood cell suspension. We have studied supernatant fluids simultaneously.

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¹ Ann. de l'Inst. Pasteur, 1897, 40, p. 177.

² Ibid., 1901, 15, p. 880.

³ Jour. Am. Med. Assn., 1903, 41, p. 962; Jour. Infect. Dis., 1907, 4, p. 207.

⁴ Centralbl. f. Bakteriol., I, O., 1913, 68, p. 602.

⁵ Jour. Immunol., 1921, 6, p. 329.

⁶ Jour. Infect. Dis., 1920, 26, p. 285.

Media.—The mediums used were: fresh meat infusion, calcium carbonate, broth having a reaction of P_H 7.4; serum broth was prepared by adding to this human or beef serum, making 5% and 20% concentrations.

Blood Cell Suspensions.—Thrice washed sheep and human corpuscles were used; after washing, the contents of the tubes were brought up to the original volume of blood with salt solution, and this was called a 100% suspension. From this a 20% suspension was prepared using salt solution.

Organisms.—Hemolytic streptococci from various sources were studied. The determination of the type of hemolytic streptococcus was made according to Brown⁷ but using human and sheep blood-agar plates.

The hemolytic properties of supernatant fluids, whole cultures and sediments were tested in tubes 1.2 cm. by 10 cm. A total volume of 2 c.c. was used with incubation for 2 hours at 37 C. in a water-bath. The tubes were undisturbed during incubation. The final concentration of red blood cells was 2%.

Supernatant fluids and sediments were obtained by centrifuging at high speed for one hour.

Sources of the Organisms.—Fourteen strains of streptococci were studied. Their sources and possible classification by the poured, 10% blood-agar plate method is shown in the table. Six of these strains were described by Clawson.⁸

SOURCE OF STREPTOCOCCI

No.	Source	Type of Streptococcus on 10% Blood-Agar Poured Plates	
		Human	Sheep
303	Puerperal sepsis.....	Beta	Beta
400	Meningitis.....	Alpha	Alpha
401	Scarlet fever, Lawrence.....	Beta	Beta
402	Clawson 36.....	Beta	Beta
403	Peritonitis case.....	Beta	Beta
404	Scarlet fever—Iowa 1.....	Beta	Beta
405	Scarlet fever—Iowa 5.....	Beta	Beta
406	Clawson 40.....	Beta	Beta
409	Scarlet fever.....	Alpha	Alpha
410	Scarlet fever.....	Alpha prime	Alpha prime
291	Clawson.....	Beta	Beta
292	Clawson.....	Beta	Beta
294	Clawson.....	Beta	Beta
295	Clawson.....	Beta	Beta

RESULTS

1. Fifteen per cent. beef and human serum calcium carbonate broth cultures. The hemolysin described by Besredka² and extensively investigated by Ruediger³ was repeatedly demonstrated. It varied in amount in young serum broth cultures from mere traces to a relatively high titer such as they describe. As a rule, it disappeared within 14 hours, but in the supernatant fluids of 3 strains, it persisted for 24 hours. Growth was good for all strains.

2. In studying plain calcium broth cultures of varying ages, some interesting results were repeatedly obtained.

⁷ Monograph 9, Rockefeller Institute for Medical Research, 1919.

⁸ Jour. Infect. Dis., 1920, 26, p. 93.

(a) Two strains, 303 and 402, possessed no hemolytic properties in whole culture, supernatant fluid or sediments for either human cells or sheep cells.

(b) The whole cultures and sediments of strains 404 and 405 were hemolytic for human cells at 8, 14 and 24 hours, whereas the supernatant fluids were not. The supernatant fluids, however, were fairly hemolytic for sheep cells at 8 hours and at 24 hours, whereas the sediments were slightly hemolytic at 8 hours and more hemolytic at 24 hours.

(c) Practically identical results were obtained when 5% beef serum calcium carbonate broth cultures were studied.

(d) Strain 409, which would be called an alpha strain if human or sheep blood-agar plates were used, produced only traces of hemolysin for human cells, whereas the sediments were not hemolytic for sheep cells, but the supernatant fluid was perceptibly hemolytic in both 8 and 18 hour cultures.

(e) Strain 410, which would be called alpha prime using human or sheep blood-agar plates, differed from 409 only in the amounts of hemolytic substances produced. It was a marked methemoglobin producer.

Since whole plain broth cultures and sediments of a number of the strains, such as 404 and 405, would produce 50% hemolysis of 2% human red blood cells whereas the supernatant fluids were not hemolytic, the following questions were studied:

1. What is the effect when amounts of cultures varying from 0.1 to 1.0 c c. are used, keeping the red blood cell concentration constant, i. e., 2% human cells?

2. What would be the result if one kept the hemolytic dose constant, using the least amount giving maximum hemolysis, and varying the concentration of human cells from 0.05% to 10%?

3. What is the effect if homologous human serum and heated beef serum are added to the tubes?

4. Will these results be the same when sheep cells are used instead of human cells?

The answer to question 1 was attempted by experiment, with the result that 0.1 c c. of whole culture or sediments gave no hemolysis; 0.2 c c., a trace; 0.3 c c., about 50%, as did 0.4 c c., 0.5 c c., etc., including 1.0 c c. One c c. of culture gave no more nor no less hemolysis than did 0.3 c c. using 2% final concentration of human red blood cells.

To answer question 2, 0.3 c.c. of culture was added to each of 11 tubes and varying amounts of 20% human red blood cell suspension and salt solution added to that; while the total volume was constant, the concentration of red cells varied. Tube 1 contained 0.05%; tube 2, 1%; tube 3, 2%; tube 4, 3%, etc., until tube 11 contained 10%. Controls of salt solution, sterile broth and equivalent concentrations of red cells laked by distilled water were made. Duplicate experiments using larger volumes were also made, and a colorimeter was used to check the results. In a few experiments, the tubes were shaken at intervals to determine whether any difference in degree of hemolysis would result.

The result showed that in tubes having red cell concentrations of 0.05%, no laking occurred; there was slight trace or none in those containing 1% concentration; 25% to 50% of 2% suspension and 50% laking in each of the remaining tubes. The amount of hemolysis increased with the concentration of red cells. In tube 11 there were apparently 10 times as many red cells hemolysed as were present in tube 1, in which no hemolysis was observed. The results also held true when 5% beef serum calcium carbonate broth cultures were used. The hemolytic supernatant fluids from this experiment containing the products of hemolysis did not possess hemolytic properties.

When 0.4 to 0.7 c.c. of homologous serum was used in lieu of that much salt solution, a noticeable inhibition of hemolysis was evident, and to a less extent when heated beef serum was added.

The supernatant fluids (streptolysin) of these cultures were lytic for sheep cells and gave the same phenomenon as described. In order to demonstrate the phenomenon, it is necessary to determine the dose of streptolysin giving only partial hemolysis of 2% concentration of cells and then keep this constant and vary the cell concentration.

It was next decided to ascertain whether these hemolytic substances were equally hemolytic for all 4 types of human cells. We obtained results that suggested that some streptococci were more hemolytic for the cells from one person than from another. Several hundred persons were typed, using the classification adopted by the Association of Immunologists in order to have several persons of the rarer types, i. e., III and IV. The results showed that there was variation in the degree of hemolysis of cells from different persons and also of the same person's corpuscles by different strains of streptococci, yet no correlation existed for certain known types. Red cells from some persons of each type were consistently more resistant to hemolysis by any of the

cultures than others. The variation in fragility is probably a large factor in this. In order that additional data might be obtained for comparison, experiments were carried out to ascertain whether a dose of saponin giving only partial hemolysis of 2% concentration of human corpuscles would be able to produce greater hemolysis depending on the concentration of red cells as shown for the hemolytic substances of streptococci. The gross appearance of the phenomenon paralleled the appearance described for hemolytic sediments and supernatant fluids.

DISCUSSION

The phenomenon which we have observed for hemolytic sediments, that a weak concentration of red cells is only slightly or not at all laked by a dose of sediment that will markedly lake a heavy concentration of red cells, would seem to be of interest, because apparently the toxicity is increased as the corpuscles are increased. This, as we shall attempt to show, may be more apparent than real. This same phenomenon can be demonstrated with supernatant fluids (streptolysin) and also with saponin, providing the proper dose is carefully selected.

In attempting to explain the phenomenon, 3 possible explanations as to the source of the hemoglobin must be considered:

1. Does the hemoglobin liberated come from a certain percentage of the cells which have been completely laked?
2. Does it come from practically all the cells, giving up a small portion each?
3. Is it a combination of both of these sources with one the outstanding factor?

When one considers the observation that in tube 11 there are 5 times as many red cells as in tube 3 and also 5 times as much hemoglobin liberated; in tube 8 there are 4 times as many cells as in tube 3 and also 4 times as much hemoglobin liberated, it would seem that the 2d or 3rd is the most likely explanation. We mention the 3rd with the 2d as the principal factor, because it would seem reasonable to suppose that there is some variation in fragility among the red cells in the blood of any person. We know that red cells are being continually destroyed by the body, and that all of the red cells are not of the same age, but we do not know what percentage of variation in fragility exists. It would seem likely that cells with increased fragility might be completely laked. It is likely that our inability to observe hemolysis in tube 1 was because of the small amount liberated.

It next would seem advisable to look at a number of possible mechanisms of hemolysis that suggest themselves:

(a) A cobra venom type of hemolysis which von Dungern and Coca⁹ and Coca¹⁰ explain on the basis of a lipase acting on the lipoids of the cell giving rise to two hemolytic substances, and which Flexner and Noguchi¹¹ explained on the basis of an amboceptor action. Their hemolytic properties seem to parallel their ability to agglutinate the red cells. The latter is not true for streptolysin.

(b) A lytic chemical substance formed by the union of some chemical substance produced by the streptococci with some chemical entity in the stroma.

(c) A saponin-like mechanism wherein a saponin-like substance is produced which brings about cell laking.

(d) A mechanism which would involve two factors, one increasing the fragility of the cell to a point where a second factor, a weak toxin, could act.

(e) A toxin alone or assisted by the products of hemolysis.

Ruediger's³ failure to demonstrate antitoxin after repeated and careful work strongly suggests that we are not dealing with a true toxin in the accepted sense.

There are a number of reasons that favor an enzyme theory. It might explain the phenomenon we describe, although one would expect a different curve of hemoglobin liberation than we observed. The temperature at which it is destroyed or inactivated is not necessarily incompatible. In addition, a similar mechanism has recently been described by Olcott and Howe¹² for the hemolytic substance of staphylococci. The work of Stevens and West¹³ is also of interest. They report the production of a lipase by hemolytic streptococci which acts at the surface of the cell. Serum, however, did not seem to protect against its action. If anti-enzymes can really be produced, as Zinsser¹⁴ and others seem to believe, then Ruediger's negative results must be considered until positive results are obtained and confirmed. If the second suggested mechanism were true, one would also have reason to expect a greater liberation of hemoglobin in the higher concentration of red cells than actually occurs.

⁹ Jour. Infect. Dis., 1912, 10, p. 57.

¹⁰ Ibid., 1915, 171, p. 351.

¹¹ Cited in Wells: Chemical Pathology, 1920, p. 223.

¹² Jour. Exper. Med., 1922, 35, p. 409.

¹³ Ibid., p. 6.

¹⁴ Infection and Resistance, 1923, p. 42.

In order to compare the hemolytic mechanism of streptococci with that of saponins, it seems advisable to mention some of the pharmacologic characteristics of the latter. Sollmann¹⁵ summarizes these as follows:

Chemic properties: Many of the typical members of the group respond to the formula $C_{10}H_{2n-8}O_{10}$. They are glucosides. They are generally not attacked by animal ferments. Physically, they possess the character of colloids; they do not dialyze; most are soluble in water; are not precipitated by moderate amounts of alcohol; but they do not dissolve in pure alcohol or in fat solvents. Many are precipitated by saturated solutions of neutral salts, especially ammonium sulphate, etc. Some behave as acids, others are neutral. . . .
Hemolytic action: Saponin hemolysis occurs in two stages, which are practically distinct; the liberation of hemoglobin, and increased permeability of the envelope. The action is probably both on the stroma and on the envelope; dissociating the former from its combination with hemoglobin and rendering the latter more permeable, thus leading to the entrance of water, swelling and "water laking."

Sollmann further states that no true antibodies occur and that serum and cholesterol have a protective action. It would appear from the foregoing that saponin represents a somewhat heterogeneous group of substances having similar pharmacologic action. One could understand that the mechanism described above would be capable of producing the hemolytic phenomenon we have described if one assumed that the saponin distributed itself as a mordant and increased the fragility of all the cells to a point where slight hemolysis resulted.

Experimentally, we have shown that saponin produces a similar phenomenon. It is supposed that saponin increases the fragility of the red cells by the removal of lipoids, but this may not necessarily be true. Ruediger's observations that streptolysin is nondialysable and fairly heat resistant, together with the protective action of serum and apparent lack of antibody production following injection, are in harmony with a saponin-like mechanism for streptolysin. His observation that streptolysin is destroyed or rendered inactive by peptic digestion and seems to be related to globulins is not in accord with the general types of saponins described. Further work may throw more light on these points. From the evidence at hand, we feel that the theory of a saponin-like mechanism of hemolysis is the more tenable explanation. This would also be in harmony with the results we obtained on the effect of hemolytic sediments and streptolysin on known types of human cells.

¹⁵ Manual of Pharmacology, 1922, p. 49.

Our work has also brought out certain apparent differences between hemolytic sediments and the soluble streptolysin, although the mechanism of action seems to be similar. A colloidal nature of the hemolytic substance might explain its apparent instability and the variable results obtained by others.

CONCLUSIONS

The similarity and differences of streptolysin and hemolytic sediments are pointed out. The evidence points toward an identical mechanism.

The phenomenon of hemoglobin liberation observed apparently indicated that the lytic substance distributes itself fairly uniformly among the red cells, causing an increased fragility and the liberation of a small amount of hemoglobin from each cell. Similar results were obtained with saponin.

Human and heated beef serums seem to exert a protective action.

In the light of Ruediger's results, this work suggests that a saponin-like mechanism of hemolysis is the most tenable of those considered.

There was found no correlation between the hemotoxins and the type of human cells laked.

DORMANCY IN BACTERIA

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The short quiescent period or lag shown by freshly planted bacterial cultures has received considerable attention. A critical review of the subject is given by Chesney.¹ The prolonged quiescence or dormancy shown by a few cells in a transplant, while of considerable more importance to the experimental bacteriologist than the lag, has escaped serious investigation until recently. Burke² described the prolonged dormancy of a small percentage of the spores of *Cl. botulinum* and suggested the importance of the subject. Eckelmann³ studied the relation of lag or slow germination of spores of soil aerobes to the failure of fractional sterilization. Her studies of the spores were not carried beyond the 11th day. She found that the spores of strains surviving fractional sterilization required from 2 to 10 days to germinate. Less attention has been given to the dormancy or slow germination of the vegetative cells of bacteria, particularly the nonspore-bearing bacteria. However, in working with single vegetative cells by the Barber technic, observation has been made that the germination of some cells is delayed beyond the latent period for the species.

This article deals with the dormancy shown by a common nonspore-bearing and a spore-bearing aerobe, and includes a brief discussion of the possible differences between lag and dormancy.

Exper. 1.—To determine the presence and duration of dormancy in a common nonspore-bearing bacillus, *Bacterium coli*, an old laboratory strain of *Bact. coli* was grown for from 18 to 24 hours on an agar slant, washed off in salt solution, sealed and shaken 5 minutes to break up the clumps. The number of organisms per c. c. was determined by a counting chamber, and a suspension was made containing an average of 2 organisms per c. c.

Inoculations were made in fresh medium by placing 0.5 c. c. of this suspension in each tube. Standard extract agar and peptone broth, each containing 0.5% glucose, were used. After inoculation, the tubes of agar were shaken before cooling in an effort to insure the single cell lodging below the surface. The tubes were incubated at 37 C. After the 2d day, the agar tubes not showing growth were sealed with wax to prevent evaporation and keep out contaminating organisms. The tubes were examined daily for the first 2 weeks and then at intervals of 3 days to a week. The examinations were continued for 6 weeks.

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¹ Jour. Exper. Med., 1916, 24, p. 387.

² Jour. Am. Med. Assn., 1919, 72, p. 89; J. Infect. Dis., 1923, 33, p. 274.

³ Centralbl. f. Bakteriol., 1917, 48, p. 141.

The dilution method of obtaining single cell cultures is sufficiently accurate for determining the presence and duration of dormancy. When the method is carefully carried out and cultures containing isolated cells and few dead organisms are used, the large majority of the agar tubes will contain but a single colony. If old cultures containing many dead cells are used, many of the tubes will remain sterile.

The results are given in table 1. Approximately 65% of the tubes showed growth at the end of the 1st day and 20% more at the end of the 2d day. From the 2d day to the 6th day, inclusive, the number of colonies developing remained uniform, about 2.4% developing each day. From the 6th to the 16th day, the number of tubes showing growth was irregular, with some skips. None of the tubes showed growth after the 16th day to the end of the observation period of 6 weeks. By that time conditions in the tubes were not favorable for growth, owing to evaporation, and the series was discarded. Since 85% of the cultures grew during the first 2 days, the mediums used may be con-

TABLE 1
RESULTS OF EXPERIMENT TO DETERMINE THE DORMANCY OF CELLS OF BACT. COLI.*

Days	Broth Tubes 241	Agar Tubes 232	Total 473	Percentage
1.....	191	127	318	65
2.....	32	64	96	20
3.....	6	6	12	2.4
4.....	3	7	10	
5.....	4	7	11	
6.....	3	8	11	
7.....	3	2	5	
8.....	..	1	1	
9.....	
10.....	..	1	1	
11.....	
12.....	1	..	1	
13.....	..	1	1	
14.....	1	4	5	
15.....	..	2	2	
16.....	..	2	2	

* The number of tubes inoculated and the number showing growth on each day are given.

sidered favorable for the growth of the average cell. Five of the 9 colonies developing on the 14th to the 16th days were subsurface colonies, which excludes the possibility of the growth being due to a contaminant dropping down from the cotton plug some time after the tubes were inoculated. The most dormant colonies were examined and found to be Bact. coli. We are justified in concluding that a small percentage of the vegetative cells of nonspore-bearing bacteria, like Bact. coli, may remain dormant for some time after the period of lag shown by the culture as a whole. After these cells start to grow, the rate of the growth of the colony appears to be normal.

No attempt was made to determine the relation of dormancy to heat resistance. It is known that some cells of Bact. coli resist the heat of pasteurization better than others. If the dormant cells are the heat resistant cells, then dormancy must be considered a factor lengthening the period between production and souring of pasteurized milk.

Exper. 2.—To determine the presence and duration of dormancy in the spores of common spore-bearing aerobes, the same method was used as in Exper. 1, except that older cultures were used to insure the presence of many spores and few vegetative cells. The vegetative cells present were too few to affect materially the results of the experiment. They could not be eliminated by heat or a disinfectant because of the possible effect on the dormancy of the spores. No broth was used.

The tubes were incubated at 37 C, and those not showing growth at the end of 48 hours were sealed with wax. The tubes were examined every day for a month, and then at intervals of 3 days to a week. Some of the tubes were under observation for 2 months, while others were observed for more than 4 months.

The results are given in table 2. These show that the spores of common aerobic bacilli may show prolonged dormancy just as do the spores of anaerobic bacteria such as *Cl. botulinum*. In dealing with spores, all surface growth must be considered as possibly due to spores becoming detached from the cotton stopper or sides of the tubes and dropping to the surface of the agar after the experiment has been started. The subsurface colonies, however, must come from spores placed in the tubes at the time the experiment is started. The most dormant colonies were identified as belonging to the species used. One subsurface colony of *B. subtilis* appeared on the 39th day of incubation and a subsurface colony of *B. megatherium* on the 90th day of incubation. Surface colonies of the 2 organisms appeared on the 109th and 120th days, respectively. We believe that with larger series of tubes the limits of dormancy for the spores of these species will be considerably extended.

TABLE 2

RESULTS OF EXPERIMENT TO DETERMINE THE PRESENCE AND DURATION OF DORMANCY OF THE SPORES OF THE COMMON AEROBES *B. SUBTILIS* AND *B. MEGATHERIUM*

<i>B. subtilis</i>			<i>B. megatherium</i>		
Day	No. Tubes Showing Growth	Type of Growth	Day	No. Tubes Showing Growth	Type of Growth
1	23		1	49	
2	63		2	47	
3	38		3	41	
4	21		4	40	
5	15		5	14	
6	15		6	10	
7	13		7	7	
9	11		9	2	
10	4		10	2	
11	1		11	2	
13	1		12	3	
14	3		13	2	
16	3	Subsurface	14	2	Subsurface
18	1	Surface	15	2	4—Subsurface
19	2	Subsurface	16	4	Subsurface
20	1	Subsurface	17	1	Subsurface
39	1	Subsurface	18	3	2—Subsurface
109	1	Surface	21	1	Subsurface
			23	1	Subsurface
			24	1	Surface
			33	1	Surface
			52	1	Surface
			90	1	Subsurface
			116	1	Surface
			120	1	Surface

DISCUSSION

The latent period or period of quiescence of viable bacteria under optimum growth conditions have been described under the two terms "lag" and "dormancy." The term "lag" is used to designate the latent period of a few hours shown by a transplanted culture during which few or none of the bacteria multiply. Dormancy is used to designate the prolonged quiescence of a few cells of the transplant after the majority

have multiplied. Thus in bacteriologic literature, the two terms have a slightly different meaning. In botanical and biologic literature, the term dormant is used to denote periods of quiescence or of rest of reproductive cells and of full-grown plants and animals. The lag shown by bacterial transplants has been the subject of considerable investigation and discussion.¹ The dormancy of bacteria, while considerably more important than lag as an interfering factor in bacteriologic research, has until recently escaped observation and investigation.²

Why cells or seeds lie quiescent or dormant under supposedly favorable conditions for growth, what factor or factors contribute to dormancy, what takes place during the dormant period and why cells or seeds of the same kind and as far as we can tell subjected to the same conditions show great variation in the length of the dormant period, remains to be determined. What part should be attributed to inherent physiologic factors; what part to mechanical difficulties, such as thick or impervious coats, and what part to transmitted injurious affects, is unknown. In speaking of the environment, it is understood that both lag and dormancy occur under optimum growth-stimulating conditions, and if any of the lag or dormancy can be attributed to the environment it is an effect carried over from a previous environment. The effect of a previous environment on dormancy may or may not be an injurious effect. It may initiate a stage in the life cycle of the organism leading to a rest period which cannot be classed as an injury. An organism may react in this manner to an unfavorable environment and avoid injury.

Two explanations have been offered to explain lag and dormancy. Chesney attributed the lag in bacterial transplants to the injurious effects of a previous environment. Burke² (1919) attributed delayed germination or dormancy of heated *Cl. botulinum* spores to "heat inhibition." Later (1923) she demonstrated similar delayed germination in unheated spores, and concluded that delayed germination was due to factors inherent within the spore, that the heating, if it had any effect, simply prolonged normal dormancy or caused a secondary dormancy. The dormant spores are the most heat resistant. She draws comparisons with the dormancy found in seeds of higher plants. Allen⁴ described the effect of "temperature shock" on bacteria in pasteurized milk. He believes that the heat shock of pasteurization attenuates some bacteria, causing them to multiply more slowly. Just what the attenuation consists of is not clear. An apparent decrease in rate of multiplication may be due to the effect of the heat on rate of multiplication, to the effect of

⁴ J. Bacteriol., 1923, 8, p. 555.

heat on the length of lag or dormancy, or to the fact that only normally dormant cells survive. Burke's experiments with the spores of *Cl. botulinum* suggest that possibly the dormant vegetative cells of *Bact. coli* are the most heat resistant.

The second explanation of lag and dormancy is that they are due to some inherent factor within the cell and not to an injury received in a previous environment. Burke² (1923) suggests that since the more dormant spores are the most heat resistant, relative permeability of the spore wall may be one of the factors determining dormancy.

Sherman and Albus⁵ suggest that the lag in freshly planted cultures is due to the physiologic necessity of the cells preparing themselves for reproduction. An analogous condition prevails in some plant seeds and strengthens this hypothesis. The known facts concerning dormancy in plants and animals deserve consideration in any discussion of the causes of lag and dormancy in bacteria. A review and discussion of the subject of dormancy in plants has been given by Crocker⁶ and Harrington.⁷ Crocker lists the possible factors affecting dormancy in plants as follows:

1. Rudimentary embryos that must mature before germination can begin.
2. Complete inhibition of water absorption.
3. Mechanical resistance to the expansion of the embryo and seed contents by enclosing structures.
4. Incasing structures interfering with oxygen absorption by the embryo and perhaps CO₂ elimination from it, resulting in the limitation of the processes dependent on these.
5. A state of dormancy in the embryo itself or some organ of it, in consequence of which it is unable to grow when naked and supplied with all ordinary germinative conditions.
6. Assumption of secondary dormancy. It is rather generally recognized that some seeds capable of immediate germination can be thrown into a secondary dormancy by a period in a germinator lacking one condition necessary for germination, or involving a substance inhibiting germination, or one hardening the colloids of the coat.

It is possible that in bacteria as in plant seeds a number of factors contribute to cause lag and dormancy, exclusive of the effect of injury received in a previous environment. If it is true that during the period of lag, as suggested by Sherman and Albus, the cells are preparing

⁵ J. Bacteriol., 1924, 9, p. 303.

⁶ Am. Jour. Botany, 1916, 3, p. 99.

⁷ Jour. Agric. Res., 1916, 6, p. 761.

themselves for rapid multiplication, why do some of them fail to reproduce for long periods after the majority have multiplied? In plant seeds, prolonged dormancy has been attributed to impervious or thick seed coats which prevent growth by failure to burst or let in oxygen or moisture—a mechanical rather than a physiologic factor. Possibly a similar situation occurs in bacterial cells. The physiologic preparation for reproduction may be retarded or growth prevented by the cell wall after the physiologic rejuvenescence is complete. The lag may be due to a physiologic factor and dormancy to a mechanical factor. The various possibilities, none of which has been eliminated, and all of which may at times operate to check multiplication, make this a promising field for future research. This is particularly true when one considers the bearing of this subject on the preservation of food and on disease.

CONCLUSIONS

Vegetative cells of nonspore-bearing bacteria may remain dormant for a number of days after the initial lag of the transplant. Cells of *Bact. coli* were found to remain dormant for 16 days. Eighty-five% of the cells were found to develop in 48 hours.

Spores of common aerobic bacteria may lie dormant for months under favorable growth conditions. Spores of *B. subtilis* were found to lie dormant for 39 days and of *B. megatherium* for 90 days. There is no evidence that this represents the limits of dormancy for the spores. A large majority of the spores develop in 4 or 5 days.

Dormancy and lag represent delayed germination of viable bacterial cells under favorable growth conditions. They are not identical phenomena and are probably due to different causes. They appear to be analogous to types of delayed growth found in higher plants.

Dormancy has an important bearing on the treatment of infections. Spores are highly resistant to disinfectants. They may lie dormant in wounds for longer periods than the applied disinfectant is effective and then germinate, and thus account for a recurrence of an infection in a wound once thought to be sterile.

Dormancy must be considered a factor in infection. It reduces the chances of infection by reducing the number of organisms that would otherwise start to grow at one time. Since the cells begin to multiply at different times, the body has an opportunity to initiate defensive reactions before all the cells develop. If dormant for a sufficient period, the organisms will be excluded from the body before development takes place.

OBSERVATIONS ON GROWTH OF COCCIDIOIDES IMMITIS

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Coccidioides immitis, a mold of the ascomycetes group, is the causal agent of coccidioidal granuloma, a disease closely resembling in many ways tuberculosis and blastomycosis.

The first human infection was observed by Posadas¹ in Buenos Aires, and was reported by him and Wernicke² in 1892. In 1896, Rixford and Gilchrist³ reported two from California and regarded the organism in the tissues as a protozoon. Ophüls and Moffit,⁴ however, demonstrated that the infecting organism is a mold, and Ophüls⁵ published an excellent description of the disease in man, reproduced it in animals, and demonstrated the growth cycle of the mold in animals and in culture mediums. Wolbach⁶ published another description of the growth cycle of the mold which agrees essentially with that of Ophüls. Since then reports of coccidioidal granuloma have been added to the literature, so that now there are about 52 reports of infection in man. Excellent résumés of the disease have been written by Hektoen,⁷ MacNeal and Taylor,⁸ Dickson,⁹ Hirsch,¹⁰ and others. Hirsch, in August, 1923, makes the last report. The mold recovered from the tissues of this host has been used in making the observations now reported.

In order to test its pathogenicity, a guinea-pig was inoculated intraperitoneally. Five weeks after inoculation, there was a suppurative inflammation of the left testicle from which the mold was recovered. The guinea-pig died in about 8 weeks with a systemic infection as described by Ophüls⁵ and others.

On ordinary mediums the mold grows well as a doubly-contoured mycelium with septums and hyphae. When the mycelium is introduced into animal tissues, some mycelial segments contract, forming doubly-contoured spheres.^{6, 8} Within each of these bodies, a hundred or more spores develop. The spores are released by the bursting of the capsule.

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¹ Rev. de Chir., 1900, 21, p. 277.

² Centralbl. f. Bakteriöl., 1892, 12, p. 859.

³ Johns Hopkins Hosp. Rep., 1896, 1, p. 209.

⁴ Phil. Med. Jour., 1900, 5, p. 1471.

⁵ Jour. Exper. Med., 1905, 6, p. 443; Jour. Am. Med. Assn., 1905, 45, p. 1291.

⁶ Jour. Med. Res., 1904, 13, p. 53.

⁷ Jour. Am. Med. Assn., 1907, 49, p. 1071.

⁸ Jour. Med. Res., 1914, 30, p. 261.

⁹ Arch. Int. Med., 1915, 16, p. 1028.

¹⁰ Jour. Am. Med. Assn., 1923, 81, p. 375.

This is the only method of reproduction in the tissues, for no mycelia grow, nor is there reproduction by budding, as in blastomyces. Endosporeulation also takes place in anaerobic ascitic fluid cultures.⁸ Wolbach⁶ found that dextrose, mannite, and saccharose are not fermented. Brown and Cummins¹¹ obtained no fermentation in mediums containing dextrose, dextrin, or lactose.

It is not unlikely that some vegetable is the intermediate host on which the organism grows in the mycelial form. It is the purpose in this study to make further observations regarding the growth limitations of the mycelium.

The mold grows well on blood agar, plain agar, beef-infusion broth, peptone broth, and in sugar-free broth. It thrives on the simplest of mediums and does not require carbohydrates. No mycelia grew in loosely stoppered tubes of nitrogen-free broth. Hence, the mold does not utilize the nitrogen of the air and does not belong to the group of nitrogen-fixing organisms. Only a few sporelike forms were found in the anaerobic ascitic fluid broth cultures. Therefore the mycelium is an obligate aerobe, and requires only a small amount of nitrogen for growth.

Since it is plausible that some intermediate host is necessary for the natural life cycle of the organism, the question of the compatibility of mycelial growth with the variations of hydrogen-ion concentration which occur in nature suggested itself. Variations in the hydrogen-ion level of the soil, according to H. W. Johnson,¹² are not commonly encountered over a wider range than from P_H of 3.62 to 9.68. Working with 7 ordinary nonpathogenic molds, he found that a P_H of from 1.6 to 3.4 on the acid side and 9 to 11.2 on the alkaline side were necessary to inhibit growth. Other studies,¹³ demonstrate that the growth of common molds is not inhibited until the P_H is as low as 0.6 to 2.0, or as high as 8.2 to 10.3.

Coccidioides immitis grows abundantly as a typical mycelium in an environment with a P_H anywhere from 2.02 to 12.13 (table 2). It did not grow when the P_H was as low as 1.07 or as high as 14. This mold, therefore, like common nonpathogenic molds, can withstand the variations in hydrogen-ion level which are commonly found in soil.

Growth of *Coccidioides immitis* in a sugar-free, nitrogen-containing medium causes a diminution of the hydrogen-ion concentration of the

¹¹ Arch. Int. Med., 1915, 15, p. 608.

¹² Iowa State College Res. Bull., 1923, 76, p. 307.

¹³ Annual Report Iowa Agricultural Station, 1920, p. 59.

medium, as recorded in table 2. Brown and Cummins¹¹ and Wolbach⁶ obtained no fermentation of several common sugars. Because of this alkaline change in the mediums inoculated with the mold and the possibility that the sugar-fermentation products were neutralized, these experiments were repeated using dextrose, maltose, lactose, and saccharose broths. Basic fuchsin was used as the indicator. The mold was allowed to grow for one month, at the end of which time no color change was observed. In order to determine whether the sugar was used and the acid neutralized by the mold-growth, a quantitative determination of the sugar present in the dextrose broth before and after inoculation was made. The first figure, using Benedict's quanti-

TABLE 1
GROWTH OF COMMON MOLDS

PH Level of Inoculated Mediums*	Growth				
	4 Days	9 Days	19 Days	26 Days	2 Months
1.02	0	0	0	0	0
2.02	0	0	Fair	Good	Good
3.0	0	Fair	Good	Good	Good
4.2	Fair	Good	Good	Good	Good
5.0	Good	Good	Good	Good	Good
6.0	Good	Good	Good	Good	Good
7.0	Good	Good	Good	Good	Good
8.0	Good	Good	Good	Good	Good
9.0	Fair	Good	Good	Good	Good
9.8	Fair	Fair	Fair	Good	Good
12.13	0	0	Fair	Fair	Fair
14.0	0	0	0	0	0

* Water, 1,800.0; peptone, 10.0; Na₂HPO₄, 14.5; KH₂PO₄, 1.4. Enlows: Pub. Hlth. Rep., 1923, 38; p. 2129.

tative test, is 80 mg. per 10 c c., and the second 80.2. The indicator was unchanged by the growth of the mold, for the addition of a small amount of dilute acetic acid produced a deep red color in the medium. Therefore, *Coccidioides immitis* does not in its growth ferment dextrose, maltose, saccharose, or lactose, nor does it destroy the indicator, basic fuchsin.

As stated, in simple sugar-free peptone mediums the hydrogen-ion concentration is diminished by the growth of the mold. The reason the mediums become more alkaline was thought most likely to be the formation of ammonia or the simpler amino compounds by the mold. V. S. Butkevich¹⁴ found that mold fungii form ammonia, utilizing both amino and amido complexes, when actively growing in nitrogen-containing mediums. S. S. Greenbaum¹⁵ in testing the biologic reactions of the

¹⁵ Jour. Infect. Dis., 1922, 31, p. 26.

¹⁴ D'Articles dédié au Prof. C. Timriazeff, 1916, p. 457; abst. Bacteriol., 1920, 4, p. 36.

molds in the trichophyton, microsporon, sporotrichum, and achorion groups, found that while no amylolytic properties were present, a proteolytic enzyme was common to all of these molds.

Tubes of sugar-free broth with the P_H adjusted to 5, 6, 7 and 8 by the colorimetric method were inoculated with the mycelium and were incubated for one month at 37 C. A tube of broth at each P_H , not inoculated, was also incubated as a control. The ammonia content was

TABLE 2
RESULT OF GROWTH OF COCCIDIODES IMMITIS IN A SUGAR-FREE, NITROGEN-CONTAINING MEDIUM

	Sugar-Free Broth		After Incubation for 1 Month at 37 C.		
	P_H	NH_3 Mg. per 100 C c.	P_H	P_H Change	NH_3 Mg. per 100 C c.
Control.....	5	0	5.0	0.0	0.00
Inoculated.....	5	0	5.7	0.7	10.00
Control.....	6	0	6.0	0.0	0.00
Inoculated.....	6	0	6.8	0.8	12.40
Control.....	7	0	7.0	0.0	0.60
Inoculated.....	7	0	7.6	0.6	9.00
Inoculated.....	8	0	8.3	0.3	5.70

then determined at each P_H in (1) the mediums not inoculated and not incubated, (2) the mediums not inoculated but incubated, and (3) the mediums inoculated and incubated. The mediums were made strongly alkaline. The ammonia present was then driven off by an air current into 10 c c. of N/100 sulphuric acid. The amount neutralized was determined by titrating the acid with N/100 sodium hydroxide, using

TABLE 3
RESULT OF ADDING DETERMINED AMOUNT OF AMMONIA FORMED IN INOCULATED MEDIUM TO NONINOCULATED MEDIUM

Medium	P_H	Procedure	Result P_H
10 c c. broth inoculated and incubated.....	6.8	Addition of 7.3 c c. of water at P_H 6.8	6.8
10 c c. broth not inoculated and not incubated...	6.0	Addition of 7.3 c c. of N/100 NH_3 *	6.8

* Equivalent of 1.24 mg. of NH_3 .

methyl red as the indicator. The results (table 2) demonstrate that ammonia is formed in the inoculated mediums, and that the greatest amount is formed in the more acid (P_H 5 and 6) mediums, those in which there is a corresponding greater change in the P_H . The P_H changes in the tubes, adjusted originally at 8, 7, and 6, were 0.3, 0.6, and 0.8, respectively, almost an arithmetical progression, using the

factor 3. The amount of ammonia produced corresponds closely to the P_H change. In the medium with the P_H of 5 there was no increase in the amount of ammonia formed over that in the medium of P_H 6, so that a P_H of about 6 is probably the critical point in the increasing production of ammonia with increased acidity.

* As a check on the ammonia determination and as evidence of the ability of the amount of ammonia formed to cause the change in the P_H of the inoculated mediums, the determined amount of ammonia formed in one of the inoculated mediums was added to the noninoculated medium (table 3). A rise in the P_H resulted which was identical with that occurring after growth of the mold.

SUMMARY

Coccidioides immitis grows well on the simplest of mediums, requiring only a small amount of nitrogen.

It is an obligate aerobe for growth in the mycelial form.

It does not belong to the group of nitrogen-fixing organisms.

It grows well in mediums with a P_H variation of from 2.02 to 12.13, and therefore can easily withstand the extremes of P_H change in the soil.

It does not by its growth ferment dextrose, maltose, saccharose, or lactose.

Ammonia is a product of its growth, greater amounts being formed in the more acid mediums until a critical point in acidity (P_H 5 to 6) is reached.

THE RELATIONSHIP OF HARD WATER TO HEALTH

I. HARD WATER AS A POSSIBLE FACTOR IN URINARY CONCRETION FORMATION

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Hard water is water that contains a considerable amount of calcium and magnesium salts. Water is usually considered soft if it carries in solution less than 75 parts per million of hardness expressed in terms of calcium carbonate. It would be moderately hard if it contained between 75 and 200 parts per million, and very hard if it contained more than 200 parts per million of hardness. Many surface waters average about 100 parts per million of hardness. Deep wells frequently contain in the neighborhood of 500 parts, but many have as much as 1,000 parts per million. In some parts of the United States, notably in North Dakota,¹ well water may contain as much as 1,800 parts per million. As is well known, temporary hardness is that portion of the calcium and magnesium which exists as bicarbonate. When the water is boiled the dissolved, carbon dioxide is expelled and the bicarbonate changed to carbonate, which is insoluble in water and precipitates.

Permanent hardness consists chiefly of sulphates and chlorides of calcium and magnesium which are not rendered insoluble by boiling.

There is considerable divergence of opinion as to whether hard water has any deleterious effect on health. It has been suspected that hard water may be injurious in three ways: by the formation of urinary concretion; by a deleterious effect on growth, general appearance, and well-being, and by the development of arteriosclerosis.

A large number of veterinarians think that water with a high content of calcium and magnesium is a factor in the causation of urinary calculi in animals. Occasionally dissent from this view is expressed. For example, F. Smith² says:

The question of hardness in water is one frequently arising, owing to the well founded prejudice against the use of hard water for horses. A very hard water, or one containing a quantity of mud or filth, acts mechanically on the membranes of the digestive canal and its results are easy of comprehension. We have been compelled in the last few years to modify

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¹ Hulbert: N. D. Agric. Exper. Station, Special Bull., 1918, 5, p. 22.

² Manual of Veterinary Hygiene, 1916, p. 17.

our views which were previously accepted as facts in connection with water and disease. It is by no means clear that any particular inorganic impurity in water accounts for goiter. It is now almost certain that while cystic calculus occurs frequently on a limestone formation, lime or hardness has practically nothing to say to its production.

Most authors on veterinary medicine hold the view that hard water is at least one of the causative factors in the formation of urinary calculi or concretions. For example, Courtenay³ says:

Cystic calculi may be caused by the use of hard water, or water containing lime, for drinking purposes, especially where the animal is worked steadily for hours at a time and not given an opportunity to urinate. In other cases a foreign substance of microscopic dimensions may be present in the bladder around which the calcareous matter may be encysted.

Quoting from James Law of Cornell University:⁴

Again the concentrated condition of the urine which predisposes to such deposits (calcareous concretions) is favored by the quantity of lime salts that may be present in the water drunk by the animal. Water that contains twenty (342 parts per million) or thirty grains per gallon (523 parts per million) of calcium sulphate or carbonate must contribute a large addition of solids to the blood and urine as compared to soft waters from which lime is absent. In this connection it is a remarkable fact that stone and gravel in the domesticated herbivora are notoriously prevalent on many limestone soils, as on the limestone formations of central and western New York, Pennsylvania, Ohio and Michigan; on the calcareous formations of Norfolk, Suffolk, and Gloucestershire in England; in Landes in France; and around Munich in Bavaria. It does not follow that the abundance of lime in the water and fodder is the main cause of the calculi, since other poisons which are operative in the same districts in causing goiter, in both man and animals, probably contribute to the trouble, yet the excess of earthy salts is probably a factor.

Law⁵ expresses a similar opinion in regard to the effect of hard water on the horse. Kinsley⁶ says:

Food or water containing excessive quantities of lime salts predispose to calculus formation.

Veterinary literature is full of similar allusions to the probability of hard water being a causative factor in the formation of urinary calculi. There is little experimental evidence for or against the theory. Geographical distribution seems to be the basis on which most of the opinions are built. The conclusion is drawn that lime must increase the formation of urinary calculi since concretions are more frequent in regions with a limestone soil. This would be explained by the increased concentration of precipitable salts in the urine, because of the increased intake of such salts in the drinking water and food.

Concretions vary markedly in chemical composition. Calcium oxalate and phosphate stones are common; calcium carbonate stones are frequent in herbivora but rare in man. Theoretically, these might be affected by the calcium intake. One would not expect other varieties, such as uric acid, urate, cystine, xanthine indigo, urosteolith, or cholesterol stones to be thus influenced.

There are some definite geographical areas where calculus is common in man. Swan,⁷ who reported 2,000 lithotomies, is of the opinion that the geographical distribution in China is due, in part at least, to the fact that people come to certain hospitals for treatment, rather than to any special composition or condition of soil or water. "Extensive limestone districts in the northern part of Kwong Tung province do not show any increase in the number of

³ Veterinary Med., 1913, p. 384.

⁴ U. S. Dept. of Agric., Bureau of Animal Industry, Special Rep., Diseases of Cattle, 1916, p. 328.

⁵ U. S. Dept. of Agric., Bureau of Animal Industry, Diseases of the Horse, 1916, p. 156.

⁶ Veterinary Path., 1914, p. 231.

⁷ Tabular Statement of 2,000 Lithotomies, 1912.

urinary calculi. Districts or counties from which our largest number of cases come are practically free from limestone formations." He concludes that "poor nutrition, bad hygiene, and a continuous disregard of the laws of health are prominent factors in the causation of stone." Swan⁸ made the interesting observation that in some cases, "a calcium oxalate stone of perhaps an inch in diameter, would be covered with a half an inch or more of sodium phosphates or urates. The closest inquiry failed to show that there had been any change in water, food, residence, or surrounding conditions of the patients which might account for the radical chemical change that had apparently suddenly taken place." The only theory he can advance is that medicinal measures resorted to by Chinese physicians had affected the chemistry of the body. This would accord with the idea that stones are the result of a disturbance of the colloidal equilibrium of the urine. These and other cases are reported by Thomson⁹ later, but he gives no additional evidence as to the rôle of water.

Cochran¹⁰ is also of the opinion that hard water is not an important factor, although there is a definite line of demarcation between certain regions in China where calculus is common and where it is uncommon.

Some experimental work has been done which has a bearing on the question. One of the earlier contributions was that of Studensky.¹¹ He used female dogs and studied the formation of concretions on foreign bodies introduced into the urinary bladder through an incision. He divided his animals into 4 groups. The 1st group was kept under as nearly normal conditions as possible, using ordinary food and a rather soft water. A small amount of lactic acid was added to the food and water of the 2d group and of oxalic acid to that of the 3rd group. The 4th group was kept under normal conditions, except that they were given water containing one part per thousand of chalk.

Various types of foreign bodies were used for each group, including polished glass beads, pellets of gutta percha, hollow jug-shaped glass objects, hollow beads with an opening at each end, rolls of lead wire, balls of gum, and irregular pieces of tin plate. In most cases, the objects remained from 1 to 3 months. In almost every instance, there was some deposition of salts on the foreign objects, especially if the surfaces were rough. The salts consisted largely of calcium and magnesium phosphates. In one instance, a bead weighing 2.4 gm. remained in the bladder of a dog for more than 3 years. A white stone was formed weighing 47.3 gm. and having the appearance of phosphates.

One of the dogs which drank chalk water lived only 3 days, and the glass bead had a thin film of calcium phosphate on it. A second dog lived 16 days; the glass bead was covered with a thin film of calcium magnesium ammonium phosphate. A third lived 2 months, and had a similar film. A 4th lived 4 months, and there was a similar but somewhat thicker layer on the glass bead and a marked precipitate on the ligature. The 5th lived 5 months after the insertion of a piece of tin plate. There was no deposit on the tin, but the ligature was slightly encrusted. Three dogs were given chalk water, and powdered bone was added to their food for 3½ months. No foreign bodies were introduced into the bladder. All were normal at necropsy.

Studensky thinks that water which contained calcium did increase the rate and amount of salt deposition on the foreign bodies, owing to increased concentration of salts in the urine. His results, however, do not show marked differences. It might seem that the addition of chalk and powdered bone to

⁸ Some Remarks on Subject of Vesical Calculus in South China, 1912.

⁹ Surg., Gynec. & Obst., 1921, 32, p. 44.

¹⁰ Personal communication.

¹¹ Deutsch. Ztschr. f. Chir., 1877, 7, p. 171.

the food and water would give results not comparable to those produced by natural hard water. Salts thus introduced would be largely insoluble except for small portions dissolved by the hydrochloric acid of the stomach. They would in all probability be excreted largely in the feces rather than by the kidneys, in which case they could have little effect on the formation of urinary calculi.

A number of investigations have an indirect bearing on the possible connection between water and formation of concretions. Ebstein and Nicolair¹² carried out an interesting piece of work on artificial production of urinary calculi. They fed oxamide to dogs, cats, rabbits, mice, goats, horses, and chickens. In a large number of cases, concretions formed of oxamide were found in the urinary tract. They conclude that oxamide causes necrosis and desquamation of epithelial cells of the bladder, which forms a nucleus for deposition of insoluble material from urine.

Tuffier¹³ followed Studensky's plan of introducing foreign bodies into the bladder of dogs. His results were negative when he used sterile glass beads. Studensky does not say his objects were sterile. The probability is that they were not. Tuffier's results were positive when he combined the methods of

TABLE 1
CALCULI IN ANIMALS AS RECORDED BY THE DRESDEN PATHOLOGICAL INSTITUTE

Species	No. of Animals Examined	No. of Cases of Calculi	Percentage of Calculi Found
Dog.....	3,301	12	0.38
Cat.....	450	1	0.22
Horse.....	2,100	11	0.50
Cattle.....	2,600	16	0.60
Sheep.....	270	5	1.90
Goats.....	90	1	1.10
Hogs.....	1,100	21	1.20

Studensky and of Ebstein and Nicolair and used either rough or septic objects in connection with feeding the oxamide. This would indicate that irritation of some kind was necessary for the formation of urinary concretions.

Klimmer¹⁴ recapitulates the subject completely. He gives statistics on the frequency of urinary calculi in animals of interest in connection with animal experimentation along this line. The following figures are taken from the records of the Dresden Pathological Institute from 1861 to 1897:

Klimmer lays great stress on geographic distribution in the occurrence of urinary calculi, emphasizing that these cases are much more abundant in limestone soil regions. He thinks that drinking water or foods rich in calcium or magnesium are important factors in the etiology of urinary concretions, because they furnish necessary building materials.

O. Kleinschmidt¹⁵ summarizes the theories of calculus formation as follows: First, there must be an organic substance to form a basis for the deposition of mineral salts. Some think that uric acid may play such a part by causing necrosis of epithelium. It is possible that catarrhal condition due to bacterial action may produce the necessary organic debris. A second theory is that a suspension of colloids in the urine may be precipitated by nucleic acid or other

¹² Exp. Erzeugung von Harnsteinen, 1891.

¹³ Arch. de Phys. norm. et pathol., 1893, p. 368.

¹⁴ Arch. f. Thierheilkunde, 1899, 25, p. 336.

¹⁵ Die Harnsteine, 1911.

acids; in short, the precipitation of a colloid by an electrolyte. A third theory is that ferments may precipitate fibrin, and the clot thus formed may serve as a nucleus for the deposition of salts. Kleinschmidt records the analysis of 56 stones in the collection of the Freiburg Pathological Institute.

Some pathologists consider the calcium intake of importance in the formation of urinary calculi in man. Abderhalden¹⁶ is of this opinion. He made a chemical analysis of a number of urinary calculi obtained from Urfa, Asia Minor. They came from people of different nationalities and ages. He found them to be largely phosphate stones, and noted that they were more common in children than in adults. He found the content of calcium and magnesium in food to be higher than in other regions; the drinking water was very hard. He gives the following analysis as that of one of the hardest waters in the region:

MgCO₃, magnesium carbonate....118.4 parts per million as CaCO

CaCO₃, calcium carbonate.....411.9 parts per million as CaCO

Abderhalden concludes that hard water is a factor in causing the large number of calculi in this region.

Keyser¹⁷ studied the effect of large amounts of calcium chloride, oxalate, lactate and phosphate on rabbits. The salts were given in some instances by stomach tube and in others by intramuscular injection.

He concludes:

Forcing lime salts in massive doses does not cause visible increase in the crystalline content of rabbits' urine. These experiments are in harmony with the views of pharmacologists that an increased ingestion of calcium salts will not lead to a significant increase of calcium in the urine. This is good evidence against a "hard water" or "lime solid" factor in the etiology of stone.

Keyser fed oxamide to animals by means of gelatine capsules, and produced urinary sand and some definite concretions. Trauma of one kidney with a needle increased the sand formation in that kidney. Slight pressure on one ureter with a rubber band increased the deposition of solids on that side.

Keyser thinks that lithiasis is due to changes in P_H , or in the colloidal equilibrium of the urine, not to an increased intake of calcium. He suggests that a protective colloidal mechanism may exist in normal urine. If this equilibrium is interfered with, precipitation of crystallizable material results.

EXPERIMENTS ON FORMATION OF CONCRETIONS

A series of experiments was undertaken in an attempt to determine whether hard water is an etiologic factor in the formation of urinary calculi. The method chosen was a study of the rate of concretion formation in animals living on hard water, as compared to those on distilled water.

Feeding Oxamide.—The work of Nicolair and Ebstein¹² and of Tuffier,¹³ suggested oxamide as a urinary irritant for the production of concretions. Two groups, each consisting of 6 normal adult rabbits, were kept under conditions as nearly identical as possible. One group

¹⁶ Ztschr. f. Physiol. chemie, 1912, 80, p. 113.

¹⁷ Arch. Surg., 1923, 6, p. 524.

received glass distilled water, the other received hard water of the following composition:

Cations in parts per million		Anions in parts per million	
Ca	217.7	HCO ₃	402.0
Mg	3.7	CO ₂	16.0
Na	169.7	SO ₄	372.1
K	35.9	Cl	55.1
Al ₂ O ₃	0.6	NO ₃	0.12
Fe ₂ O ₃	0.5	NO ₂	0.0
SiO ₂	8.0		

TABLE 2
RABBIT SERIES 1. FED OXAMIDE

Length of Experiment in Days	Distilled Water						Hard Water						Average Wt. of Hard Water Rabbits, Gm.	Average Wt. of Distil. Water Rabbits, Gm.
	Rabbit 1, Gm.	Rabbit 2, Gm.	Rabbit 3, Gm.	Rabbit 4, Gm.	Rabbit 5, Gm.	Rabbit 6, Gm.	Rabbit 1, Gm.	Rabbit 2, Gm.	Rabbit 3, Gm.	Rabbit 4, Gm.	Rabbit 5, Gm.	Rabbit 6, Gm.		
0	2,820	2,530	2,825	2,460	2,060	2,215	2,650	2,220	2,450	3,170	1,975	2,220	2,447	2,418
31	2,270	2,280	1,950	2,330	1,750	1,515	2,320	2,200	2,225	2,650	Dead	2,010	2,280	2,016
38	2,150	2,370	2,200	2,120	1,830	1,380	1,865	2,375	Dead	2,455	2,080	2,025	2,010
45	2,285	2,250	2,240	2,290	1,925	1,385	Dead	2,410	2,480	2,060	2,316	2,060
52	2,410	1,435	2,250	2,380	2,025	1,240	2,175	Dead	2,175	2,175	2,125
59	2,445	2,440	2,440	2,400	2,200	Dead	1,575	1,975	1,775	2,385
66	2,540	2,409	2,465	2,420	2,075	Dead	1,960	1,960	2,395
80	2,400	2,485	2,600	2,275	2,065	Dead	2,353
108	2,750	2,775	2,700	2,150	2,100	2,495

The calcium and magnesium calculated as carbonates would be as follows:

Calcium carbonate	519.2 parts per million
Magnesium carbonate	13.0 parts per million

This is a larger amount of calcium and a smaller amount of magnesium than Abderhalden¹⁶ found in the water at Urfa. His figures are as follows:

Calcium carbonate	411.3 parts per million
Magnesium carbonate	118.4 parts per million

Three grams of oxamide per rabbit per day were moistened with hard water for the hard water group and with distilled water for the distilled water group, and mixed with the oats fed to the animals. The diet consisted of oats and carrots. Oxamide was prepared by the following method: Equivalent proportions of anhydrous oxalic acid and 95% ethyl alcohol were heated for 4 hours on a boiling water bath using a reflux condenser. The resulting ethyl oxalate was treated with an equivalent quantity of ammonium hydroxide. The copious precipitate

of oxamide was washed thoroughly with water, recrystallized from hot alcohol, and again washed well with water. The purity of the final product was ascertained by a nitrogen determination using the Kjeldahl-Gunning method. Nitrogen by theory is 31.80%. We found 21.90% and 32.00%.

During the first 30 days, all of the rabbits lost weight at about an equal rate. Table 2 shows the progress of the experiment.

No definite concretions were found in either group, but 2 of the hard water rabbits showed an inflammation of the urinary bladder with solid material precipitated but not aggregated in the urine. No irritation of the urinary organs or precipitation of solid matter in the urine was observed in any of the distilled water animals. This might sug-

TABLE 3
WEIGHT OF SOLIDS FOUND IN BLADDERS OF ANIMALS GIVEN DISTILLED WATER
AND HARD WATER

Rabbit No.	Distilled Water			Hard Water		
	Length of Experiment, Days	Volume of Urine, C c.	Weight of Solids in the Bladder, Gm.	Length of Experiment, Days	Volume of Urine, C c.	Weight of Solids in the Bladder, Gm.
1.....	14	5	0.042	20	4	0.065
2.....	15	6	0.065	37	3	0.245
3.....	47	17	0.062	47	7	0.975
4.....	47	31	1.180	47	3	0.180
5.....	47	30	0.780	47	10	0.255
6.....	47	6	0.640	47	26	1.750
Total.....	..	95	2.769	..	53	3.470

gest that hard water combined with other factors is capable of producing what could be called a preconcretion condition. In other words, the results might suggest that hard water could be an additive factor in the causation of urinary concretions, provided other necessary factors were present; but the evidence is slight.

Administration of Oxamide in Capsules.—Keyser's¹⁷ investigations suggested the administration of oxamide to rabbits by means of gelatine capsules. The method is more accurate than mixing the oxamide with the food. Each rabbit was given a capsule containing approximately 0.5 gm. of oxamide daily. With this exception, the experiment was identical with the preceding one. The animals were kept on the given water for 2 weeks to accustom them to it before starting with the oxamide (table 2).

There was a wide variation in the amount of solids in the bladder of the animals in each group, although the total quantity of solids was somewhat greater in the animals given hard water. The deviation from the mean is so great that one would not be justified in placing much significance on the results. At any rate, the evidence implicating hard water is not convincing.

The Production of Concretions by Foreign Bodies.—A sterile glass bead 4 mm. in diameter was placed in the left kidney of each of 4 dogs. Rigid asepsis was observed.

As far as this work goes, it would suggest that concretions are formed as readily on a distilled water as on a hard water intake, since a definite concretion was formed on the bead in the dog which lived 215 days under a distilled water regime.

TABLE 4
FORMATION OF CONCRETIONS ON A HARD WATER AND ON A DISTILLED WATER INTAKE

	Diet	Water	Length of Exper. in Days	Fate of Animal	Encrus- tation
Dog 1.....	Mixed	Hard	47	Died	Slight
Dog 2.....	Mixed	Hard	214	Lost
Dog 3.....	Mixed	Distilled	12	Died	None
Dog 4.....	Mixed	Distilled	215	Killed	4 mm. thick

Glass Beads in the Kidneys of Rats.—A sterile glass cylinder about 2 mm. long and 1 mm. in diameter was inserted in the left kidney of each of 24 rats. The rats were young, weighing from 50 to 60 gm. at operation. They were divided into 4 groups: (1) a low calcium diet and distilled water, (2) a low calcium diet and hard water, (3) a high calcium diet and distilled water and (4) a high calcium diet with hard water.

The basal diet was prepared according to the following formula:

	Gm. or C c.
Casein, washed	175
Potato starch	500
Butter	100
Autolysed yeast, dry weight.....	50
Cod liver oil.....	50
Orange juice	50

Five % of pulped paper was added to provide roughness. This diet is a modification of the one used by Luce¹⁸ as a diet for rats. It was

¹⁸ Jour. Path. & Bacteriol., 1923, 26, p. 200.

modified by adding more yeast, because it was found that rats did not reproduce well, and that chickens did not live with a smaller amount.

The casein and paper were washed with hydrochloric acid and at least 10 changes of distilled water or until only faint traces of calcium remained.

For the high calcium diet, 5% of the salt mixture of Steenbock and Nelson¹⁹ was used.

	Molecules	Grams
NaCl	4	233.6
MgSO ₄ , 7H ₂ O	1	246.0
NaH ₂ PO ₄ , 12H ₂ O	1	358.0
K ₂ HPO ₄	4	696.0
Ca ₂ H ₂ (PO ₄) ₂ , 4H ₂ O	2	698.0
Ca(C ₃ H ₅ O ₃) ₂ , 5H ₂ O	0.5	154.0
Fe(C ₆ H ₅ O ₇) ₂ , 6H ₂ O	0.1	59.8
KI	0.01	1.6

For the low calcium diet, all of the calcium was omitted from the foregoing formula. A mixture of 3.3% of the remaining salts was added to the basal diet so that the other salts were equivalent.

Most of the rats remained alive at the end of 120 days, when they were killed and necropsies performed. Three rats survived in group 1, four in group 2, four in group 3, and six in group 4. The others died from diarrhea or unknown causes while the animals were on test. Necropsy was performed on each rat at death. The glass bead was recovered in all cases except one, that of a member of group 1. In about half of the animals, the bead was found in or partially in the pelvis of the kidney. In the others, it was embedded among the tubules in the parenchyma. The kidneys had all completely healed and were apparently functioning normally.

In no case was any salt deposition found on the bead. Hence no conclusions can be drawn as to the effect of hard water on the rate of concretion formation. Why deposition did not occur as in the case of the dogs is not known. Possibly concretions do not form readily in the rat. At any rate, there is no evidence in favor of the theory that hard water is a causative factor, since even with a high calcium diet and hard water no stones were formed about the glass beads.

Cartilage in the Bladder of Rabbits.—Cartilage was cut in cubes 6 mm. on a side, sterilized with 80% alcohol, and the alcohol removed with sterile isotonic sodium chloride solution. One cube was placed in the urinary bladder of each of 2 rabbits. The suprapubic route was

¹⁹ J. Biol. Chem., 1923, 56, p. 355.

used, the bladder being sutured with fine silk. Rigid aseptic precautions were observed throughout and the skin incision coated with collodion.

Both rabbits recovered from the operation promptly, but 1 died from diarrhea after 6 days. The cartilage was intact and covered with a layer of salts about 1 mm. thick. This indicated that the technic might be feasible.

In a similar manner, cartilage cubes were put in the bladder of 12 rabbits. They were divided into 4 groups and placed on the same diets and waters prescribed for the rats on which the kidney had been operated, except that 25% of oats was added to all diets. Preliminary experiments indicated that the digestive system of the rabbit was not adapted to such a concentrated diet. The animals almost invariably died, apparently of intestinal stasis. The stomach and cecum were

TABLE 5
RESULTS OF EXPERIMENTS ON RABBITS IN THE BLADDERS OF WHICH CARTILAGE CUBES
WERE PLACED

Rabbits	Diet	Water	Days on Test	Dry Weight of Concretion, Gm.	Dry Weight of Ash, Gm.
1.....	High Ca	Hard	60	0.160	0.060
2.....	High Ca	Hard	51	0.616	0.359
3.....	High Ca	Hard	60	0	0
4.....	High Ca	Distilled	60	0	0
5.....	High Ca	Distilled	60	0	0
6.....	High Ca	Distilled	60	0	0
7.....	Low Ca	Hard	49	0	0
8.....	Low Ca	Hard	60	0.064	0.011
9.....	Low Ca	Hard	60	0	0
10.....	Low Ca	Distilled	37	0	0
11.....	Low Ca	Distilled	47	0.121	0.052
12.....	Low Ca	Distilled	60	0	0

filled with a firm but somewhat pasty mass. The remainder of the digestive tract was empty. The animal was greatly emaciated. The trouble seemed to be due to a lack of crude fiber, hence the addition of oats. On this regime the animals did fairly well.

The largest concretion came from a rabbit which had been on a high calcium diet and hard water, but one from a rabbit which had been in the same cage under the same conditions was very much smaller. The smallest of all came from a rabbit on a low calcium diet but hard water. The concretion from one on a low calcium diet and distilled water was fairly large.

There seems to be nothing in this experiment which would prove that hard water favors the rate of concretion formation.

SUMMARY

Oxamide fed to rabbits produced only a slightly larger amount of solid material in the urinary bladder when the rabbits received hard water than when they were given distilled water.

Glass beads placed in the kidneys of dogs produced concretions with both a hard and a distilled water intake.

Glass beads in the kidneys of albino rats failed to produce concretions with either type of water.

No definite relationship was observed between hardness of the water intake and the amount of calcification of cartilage blocks in the bladders of rabbits in conjunction with either a high or a low calcium diet.

No evidence has been found proving hard water to be an etiologic factor in urinary concretion formation.

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